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BARBARA DAWN FANNING

Date of Birth — Date de naissance	Country of Birth — Lieu de naissance
08/09/54	CANADA

Permanent Address — Résidence fixe

#3807, 88 BLOOR ST. E.
TORONTO, ONTARIO
M4W 3G9

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IN VITRO SENSITIZATION OF HUMAN LYMPHOCYTES TO SHEEP ERYTHROCYTES

by

© BARBARA FANNING

A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled IN VITRO SENSITIZATION OF HUMAN LYMPHOCYTES TO SHEEP ERYTHROCYTES submitted by BARBARA FANNING in partial fulfilment of the requirements for the degree of MASTER OF SCIENCE in IMMUNOLOGY in the DEPARTMENT OF MEDICINE.

John B. J. Donelson

Supervisor

T.A. McPherson

H.F. Pabst

F. Rozsa

F. Rozsa

A.R.E. Shaw

Date April 15th 1981

ABSTRACT

The *in vitro* culture and assay systems of Dosch and Gelfand (1976), were used to induce and measure the plaque forming cell (PFC) response to sheep erythrocytes (SRBC) by human lymphocytes (PBL).

The response was standardized in 49 normal individuals; the optimal antigen dose being 10^8 SRBC/culture, the optimal lymphocyte dose being 0.5×10^6 or 1.0×10^6 per 10 mL. culture, and the peak response was observed on day six. Using 10^8 SRBC to stimulate freshly prepared PBL the results from 27 individuals were 663 ± 324 PFC/10^6 viable cells on day 6 when 0.5×10^6 PBL were cultured and 623 ± 401 PFC/10^6 viable cells when 1.0×10^6 PBL were cultured. These results represent the first characterization of an *in vitro* generated immune response in normal individuals.

The day to day variation in responses observed from one individual was often much greater than that observed between individuals.

After cryopreservation, PBL responses became more reproducible and higher. When cryopreserved lymphocytes from the same 27 individuals were stimulated in cultures containing 0.5×10^6 or 1.0×10^6 PBL/culture in the presence of 10^8 SRBC, the PFC numbers obtained were 1078 ± 307 and 951 ± 381/10^6 cells, respectively.

When reclaimed lymphocytes were mixed with freshly prepared autologous PBL in varying proportions, increased responses were observed in comparison to results from cultures of fresh lymphocytes. The results indicate that some of the variability observed with the fresh PBL responses from normal individuals may be due to the presence of variable numbers of suppressor cells which are selectively removed due to the treatment involved in lymphocyte freezing.

Pre-incubation of PBL for 1, 3 or 6 days with or without antigen allowed the induction of suppressor cells which could suppress the responses of freshly reclaimed, autologous PBL. Cell separation experiments showed that this cell was a T lymphocyte. B cells and monocytes treated in the same manner suppressed the responses of autologous PBL to a considerably lesser degree. The addition of increasing numbers of pre-incubated T cells showed that as few as 2×10^5 of these cells would give 50% suppression of the response from cultures of 0.5×10^6 freshly reclaimed, autologous PBL.

Further studies are necessary before *in vivo* functions of these suppressor T cells may be defined.

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TABLE OF CONTENTS

Title Page..... ii

Approval Sheet..... iii

Abstract..... iv

Acknowledgements..... vi

Table of Contents..... vii

List of Tables..... xi

List of Figures..... xii

List of Abbreviations..... xiv

CHAPTER I INTRODUCTION AND LITERATURE REVIEW..... 1

A. Introduction..... 1

B. The Major Histocompatibility Complex..... 5

C. The Major Histocompatibility Complex in Mice..... 6

1. The I Region of H-2..... 6

D. The Major Histocompatibility Complex of Man..... 10

1. Immune Response Genes and Disease Susceptibility in Humans..... 11

E. Techniques for Study of *In Vitro* Generated Antibody Formation with Mice..... 14

F. Results From Early Murine System Experiments..... 16

G. Results From Early Work With Human Lymphocytes..... 19

1. Tonsillar Lymphocytes..... 19
2. Peripheral Blood Lymphocytes..... 20
3. Variability in the Response to SRBC by One Individual..... 22

H. Development of Suppressor Cell Analysis by PFC Responses With Mice..... 25

I. Development of Suppressor Cell Analysis by PFC Responses with Humans..... 28

CHAPTER II MATERIALS AND METHODS..... 33

A. Preparation of Peripheral Blood Leukocytes (PBL)..... 33

B. Separation of Different Subpopulations of PBL..... 33

1. Preparation of T Lymphocytes by Sheep Erythrocyte (SRBC) Rosetting..... 34
   a. Preparation of Aminoethylisothiouronium Hydro bromide (AET) treated SRBC..... 34
   b. Preparation of SRBC Absorbed Fetal Calf Serum (FCS)..... 34
   c. E-Rosetting Technique and Separation of Rosetted Cells From Non-Rosetted Cells..... 35
2. Preparation of Monocytes..... 35
3. Nylon Wool Separation of T and B Lymphocytes..... 36

C. Tests for Checking the Purity of Subpopulations of PBL..... 36

1. Rerosetting with AET Treated SRBC..... 36
2. Percent Killing with Anti B-Cell Serum..... 36

D. Freezing and Reclaiming of Lymphocytes..... 37

E. *In Vitro* Sensitization of PBL to Sheep Erythrocytes..... 37

1. The Culture Medium..... 37
2. Human AB Serum Pool..... 37
3. Sheep Erythrocyte Antigen..... 37
4. Culture Protocol..... 38

F. Plaque Forming Cell Assay..... 38

G. Calculation of Results..... 39

H. Controls..... 39

I. Comparison of Responses From Fresh and Reclaimed Cells..... 39

J. Addition of Pre-incubated Lymphocytes to Autologous Cultures..... 40

K. Statistical Methods..... 40

CHAPTER III RESULTS..... 42

A. Control PFC Results..... 42

B. Purity of Cell Separations..... 42

1. T Cell Enriched..... 42
2. B Cell Enriched..... 42
3. Monocytes..... 42

C. Kinetics of the PFC Response..... 42

D. Effect of Varying Antigen Dose on the PFC Response..... 42

E. Effect of Varying Lymphocyte Dose on the PFC Response..... 43

F. Effect of Volume Variation on the PFC Response..... 43

G. Effect of Lymphocyte Treatment with Mitomycin C on the PFC Response..... 43

H. Variation Between Experiments..... 44

I. Variation Within an Experiment..... 44

J. Day to Day Variation..... 44

K. Effect of Lymphocyte Proliferation in Inducing Cultures on PFC Results..... 45

L. Effect of Adherent Cell Removal on the PFC Results..... 45

M. Requirement for T and B Lymphocytes..... 45

N. Comparison of T/B Cell Separations by SRBC Rosetting and by Nylon Wool Column Adherence..... 45

O. Studies with Frozen, Reclaimed Lymphocytes..... 46

P. Effect of Addition of Pre-incubated Cells to Autologous Cultures on the PFC Response..... 47

1. Pre-incubation of PBL..... 47
2. Pre-incubation of T Cells, B Cells, and Monocytes..... 48
3. Quantitation of T Suppressor Cell Activity..... 48
4. Varying Media in the Primary Culture..... 49

CHAPTER IV DISCUSSION AND CONCLUSIONS 97

BIBLIOGRAPHY..... 110

APPENDICES..... 125

1. Kinetics of the PFC Response..... 125

2. Kinetics of the PFC Response..... 127

3. Kinetics of the PFC Response..... 129

4. Effect of Varying Antigen Dose on the PFC Response..... 131

5. Effect of Varying Antigen Dose on the PFC Response..... 133

6. Effect of Varying Antigen Dose on the PFC Response..... 135

7. Effect of Varying Antigen Dose on the PFC Response..... 137

8. Effect of Varying Antigen Dose on the PFC Response..... 139

9. Effect of Varying Antigen Dose on the PFC Response..... 141

10. Effect of Varying Antigen Dose on the PFC Response..... 143

11. Effect of Varying Antigen Dose on the PFC Response..... 145

12. Plaques Seen in One Well Under 2.5 x Objective..... 147

13. Plaques Seen in One Well Under 10 x Objective..... 149

LIST OF TABLES

Table 1. Summary of Suppressor Cell References..... 32

Table 2. Purity of Cell Preparations..... 87

Table 3. Effect of Lymphocyte Treatment with 25 µg Mitomycin C on the PFC Response..... 88

Table 4. Variation Between Experiments..... 89

Table 5. Variation Within an Experiment..... 89

Table 6. Day to Day Variation in 20 Individuals, Each Tested on 4 Occasions..... 90

Table 7. Comparison of T/B Cell Separations by SRBC-Rosetting and by Nylon Wool Column Adherence..... 91

Table 8. PFC/10^6 Cells and Proliferation Indices vs % T cells..... 92

Table 9. Variation Between Experiments - Reclaimed Cells from Different Bleeds..... 93

Table 10. Effects of DMSO Treatment and Freezing on PFC Responses by PBL..... 94

Table 11. Suppressor Indices in Cultures with Added Pre-incubated T Cells..... 95

Table 12. Addition of T Cells Pre-incubated Without Antigen in Various Media to Cultures of Autologous PBL..... 96

LIST OF FIGURES

Figure 1. Kinetics of the PFC Response..... 51

Figure 2. Effect of Varying Antigen Dose on the PFC Response..... 53

Figure 3. Effect of Varying Antigen Dose on the PFC Response..... 55

Figure 4. Effect of Varying Lymphocyte Dose on the PFC Response..... 57

Figure 5. Effect of Volume Variation on the PFC Response..... 59

Figure 6. Effect of Lymphocyte Proliferation in "Inducing" Cultures on PFC Results..... 61

Figure 7. Effect of Lymphocyte Proliferation in "Inducing" Cultures on PFC Results..... 63

Figure 8. Effect of Adherent Cell Removal on PFC Results..... 65

Figure 9. Effect of Lymphocyte Proliferation in "Inducing" Cultures on PFC Results..... 67

Figure 10. Comparison of PFC Responses From Fresh and Freshly Reclaimed Lymphocytes..... 69

Figure 11. Effect of Adding Freshly Reclaimed, Autologous PBL to Cultures of Fresh PBL, on the PFC Response..... 71

Figure 12. Effect of Adding Freshly Reclaimed, Autologous PBL to Cultures of Fresh PBL, on the PFC Response..... 73

Figure 13. Effect of the Addition of Pre-incubated Cells to an Autologous Culture on the PFC Response..... 75

Figure 14. Effect of the Addition of Pre-incubated Cells to an Autologous Culture on the PFC Response..... 77

Figure 15. Effect of the Addition of Pre-incubated Cells to an Autologous Culture on the PFC Response..... 79

Figure 16. Effect of the Addition of Pre-incubated Cells to an Autologous Culture on the PFC Response..... 81

Figure 17. Effect of the Addition of Pre-incubated Cells to an Autologous Culture on the PFC Response..... 83

Figure 18. Addition of T Cells to Autologous Cultures..... 85

Abbreviations Used

1.	ABS	-	human AB serum
2.	AET	-	aminoethylisothiouronium hydrobromide
3.	CON A	-	concanavalin A
4.	DR	-	D region related
5.	DMSO	-	dimethyl Sulfoxide
6.	DNP	-	dinitrophenol
7.	DNP-MON	-	dinitrophenol monomeric flagellin
8.	E-RFC	-	erythrocyte-rosette forming cell
9.	FCS	-	fetal calf serum
10.	F-H	-	ficoll-hypaque
11.	H-2	-	major histocompatibility complex of the mouse
12.	HLA	-	human leukocyte antigens
13.	HRBC	-	horse red blood cells
14.	Ia	-	I region associated
15.	IR	-	immune response
16.	KLH	-	keyhole limpet hemocyanin
17.	LD	-	lymphocyte defined
18.	MHC	-	major histocompatibility complex
19	MLC	-	mixed lymphocyte culture
20.	ORBC	-	ox red blood cells
21.	PBL	-	peripheral blood lymphocytes
22.	PFC	-	plaque forming cells
23.	PHA	-	phytohemagglutinin
24.	PLL	-	poly-L-lysine
25.	PWM	-	pokeweed mitogen
26.	RNA	-	ribonucleic acid
27.	SD	-	serologically defined
28.	SRBC	-	sheep red blood cells
29.	TG	-	T cells bearing a receptor for IgG
30.	TM	-	T cells bearing a receptor for IgM
31.	TNP-PAA	-	trinitrophenol polyacrylamide
32.	T-resis.	-	theophylline-resistant
33.	T-sens.	-	theophylline -sensitive

I. Introduction and Literature Review

A. Introduction

Some immune response (Ir) genes in humans are believed to be contained in, or linked to, the major histocompatibility region on chromosome six. The strongest evidence for this association comes from the correlation observed between the human major histocompatibility system (HLA) and various diseases. (Svejgaard et al, 1975). Kato et al (1980) reported an increased frequency of HLA-B15 and decreased frequency of Bw22 in patients with congenital rubella syndrome. HLA-B15 was associated with high antibody responsiveness to rubella vaccine and Bw22 was one of the HLA antigens showing low antibody responsiveness.

Studies of different levels of immune response to certain antigens have been relatively infrequent. Black et al (1976) demonstrated that high responses to ragweed antigen are associated with B7.

In the human in vitro culture systems so far studied, individual variation in the response to trinitrophenol-polyacrylamide (TNP-PAA) conjugates has been reported. (Galanaud et al, 1977).

The measurement of immune responses to various antigens in vitro has been hampered by the lack of a reproducible technique, so the genetic control of antibody production in humans has remained unclear. (Fauci and Pratt, 1976).

Studies of immune responsiveness in autoimmune disease (Dosch et al, 1978; Waldmann and Broder, 1977) have begun to give some insight into the mechanisms of an altered immune response but to date the genetic control of a normal response has not been studied.

In the mouse model, responsiveness to a particular antigen varies between inbred strains, (Shreffler and David, 1975). Use of congenic strains has shown that responsiveness is controlled by genes in the I region of H-2, and that some antigens under Ir gene control require the presence of responder alleles at two distinct I region loci (gene complementation). (Dorf et al, 1975).

Functional analyses of murine Ia antigens E and C; and I-A has indicated that they could be the products of Ir genes (Uhr et al, 1979). There is evidence that the gene products of I-E and I-C subregions are expressed on B cells and macrophages. (Frelinger et al, 1978) and that both products play critical roles in cell interaction. (McDevitt, 1978).

Sequence analysis of human DR antigens indicates a homology with the murine I-E/C subregion controlled alloantigens. It is probable that both alpha and beta subunits of DR antigens are controlled by HLA - linked loci. There are also loci linked to HLA which may control one of the Ia antigen subunits in humans. (Tosi et al, 1978).

This study was initiated to investigate the variation in the response to SRBC among normal individuals. This antigen was chosen because of its known immunogenicity in humans, (Dosch and Gelfand, 1976; Galanaud et al, 1977; Luzzati et al, 1976) and its complexity. The antigen or pathogens responsible for many human diseases are complex, so when looking for an association between immune responsiveness and the genetic control of disease, it is reasonable to choose such a multi-determinant antigen (Klein, 1975).

The difficulties with this antigen are (1) varying surface characteristics with each batch, including variable immunogenicity

and complement sensitivity (Cunningham and Pilarski, 1974) and, (2) cross-reactivity with other xenogenic antigens that an individual may have encountered. (Mishell and Dutton, 1967).

A plaque assay was chosen to enumerate single antibody forming cells. (Jerne and Nordin, 1963). It's use in the mouse system to characterize the immune response to many simple and complex antigens is extensive. (McDevitt and Landy, 1972).

At the outset of this study, it was postulated that each individual would respond by forming a given number of plaques per million lymphocytes in culture. (Dosch and Gelfand, 1976). It was further thought that if one group of individuals responded by making a significantly higher number of plaques than a second group, these two groups might show significant association with different HLA antigens on the population level, or with haplotypes in family analysis.

This hypothesis stems from the results of work within the mouse model, where, using congenic strains the response was found to be controlled by loci within H-2. (Sabolovic et al, 1971). The H-2 differences could be acting at several levels of the immune response, either at recognition of the antigen, processing of the antigen, or at the level of antibody formation. These authors reported the variation at the level of IgM antibody production, as have others. (Klein, 1975; McDevitt and Chinitz, 1969; McDevitt and Landy, 1972).

However, our initial exploratory experiments using the PFC technique with normal human PBL demonstrated such a large amount of day to day variability that a distinction between "high" and "low" responders could not be made. Several experimental variables were

explored in order to be able to distinguish between high and low responders (antigen and/or lymphocyte dose effect, kinetics of the response) but these experiments only established the optimal conditions for obtaining reproducible PFC counts for all individuals. Still, day to day variation of the reactivity of the cells of a given individual remained higher than that between individuals.

Thus, our main efforts were concentrated on finding the explanation for this variability.

Studies in this thesis have led us to hypothesize that some of the day to day variability in in vitro responses to SRBC observed among normal individuals may be explained by varying numbers of suppressor cells present in the T lymphocyte population.

This observation led us to a further series of experiments where we attempted in vitro induction of suppressor cells in this system, as previously described by (Heijnen et al, 1979) for PFC responses to SRBC and ovalbumin. (Shore et al, 1978; Uytde Haag et al, 1978).

Conditions for suppressor cell induction and characterization of the cell populations involved in this suppression phenomenon are described in this thesis.

B. The Major Histocompatibility Complex

All mammals possess one very similarly organized chromosomal complex that determines components of major importance for individual diversity,that is instrumental in the control of immune responses to most foreign antigens, that determines several cell surface structures, and that may be important in the control of cell interactions such as those involved in morphogenesis. (Bach and Van Rood, 1976).

The major histocompatibility complex (MHC) is controlled by a number of very closely linked genes that show great polymorphism and are expressed codominantly. The gene products include cell membrane molecules showing allogenic variation (HLA, H-2, etc.) complement factors, (C_2, C_4, factor B), and products of immune response genes controlling the response to thymus dependent antigens. (Thorsby, 1979).

C. The Major Histocompatibility Complex in Mice

The H-2 system, the murine major histocompatibility complex, is reported to have control over some 60 traits. These range from reactivity with sheep erythrocytes to T-B cell cooperation and androgen levels. Of special interest to immunologists are those factors influencing resistance or susceptibility to viral infections, autoimmune disease, and neoplastic disease. (Klein, 1978).

Mapping of the genetic distance between loci and the sequencing of loci within the complex is achieved by several techniques. A loss of an H-2 associated trait, and accompanying the loss, through crossing over, of a part of the H-2 complex is interpreted as evidence that the missing part controls the particular trait. Genes controlling several traits have been mapped into the same regions of the H-2 complex. A region could control just one locus, or several additional loci about which we may have no information. If a point mutation alters several traits, then the traits must be controlled by the same locus.

1. The I region of H-2

Within the I region of H-2, the Ir-IA, IB, and IC subregions are each defined by genes which control the immune response to a variety of antigens. (Shreffler and David, 1975). The Ir-IA, IB, and IC subregions are defined by alloantigens (David et al, 1976) and the IJ subregion by suppressive function and suppressor factors. (Murphy et al, 1976). In addition, all the subregions, with the possible exception of Ir-IB, are associated with surface markers expressed on immunocytes called Ia antigens. Anti-Ia sera can block the stimulating cell in the mixed lymphocyte reaction, (Meo et al,1975)

the acceptor site of the B cell for T cell helper factor, (Taussig et al, 1975) and the binding of aggregated immunoglobulin to FC receptors. (Dickler and Sachs, 1974).

One of the exceptions to the homology between HLA and H-2 is the mapping of HLA-D outside the segment coding for the serologically defined antigens. The functions of the D and I regions may be homologous but there is some suggestion that some of the B cell alloantigens in man are coded for by determinants close to HLA-B as well as HLA-D. (Dupont et al, 1976).

Most genes which determine immune responses are dominant, so, to study their inheritance, inbred strains in which inbreeding has fixed many non or low responder genes are used.

Specific immune responses to certain antigens of restricted heterogeneity were shown by Pinchuck and Maurer (1965) to be controlled by dominant, autosomal genes. Information clearly defining an immune response gene came from studies on the response of inbred strains of mice to the branched, synthetic polypeptide antigen (T,G)-A--L, which bears a restricted range of antigenic determinants. (McDevitt and Sela, 1965).

<u>In vivo</u> immunization of congenic strains B10 and B10-A(5R) by Markman and Dickler(1980)showed that the immune response gene(s) for (T,G)-A--L is located in the I-A subregion of the H-2 complex. These data are consistent with the hypothesis that Ia antigens are Ir gene products.

Each H-2 haplotype shows a different pattern of responses and non-responses to a large panel of simple and complex antigens (Shreffler and David, 1975). This indicates a high degree of polymorphism in Ir

genes, due to multiple alleles, multiple genetic loci, or both. The pattern of responses by haplotype is different for each antigen.

This strongly suggests the existence of multiple Ir genes, with each gene determining a different response. (Vitteta and Capra, 1978).

One model for the mechanism of histocompatibility-linked Ir gene function is that these genes control the Ia molecules on macrophages and B cells. (Benacerraf and Germain, 1978). These molecules would interact with T dependent antigens such that an Ia antigen specific stimulus develops which is able to activate only specific helper, delayed type hypersensitivity, or proliferative T cells. Alternatively, the Ir genes may be primarily expressed on T cells and be concerned with the production of specific helper and suppressor factors, which bear determinants coded for by loci in the I-A and I-J subregions respectively.

The distinction between high and low responder strains of mice may be made only when antigen dose, route of injection, time of immunization, molecular weight of the antigen, and method of assay for immune responsiveness are clearly defined. For example, (Klein, 1975) at a low dose of antigen C57 and CBA strains are both low responders, at a high dose C57 mice give a higher response but at an intermediate dose the strain difference is the largest.

Immune response genes, for example Ir-IA, may distinguish very well between cross reacting antigens. These genes may act on a particular immunoglobulin class, for example Ir-IA controls the secondary but not the primary immune response to (T,G)-A--L. The low responder's inability to mount a secondary reaction seems to be linked to the presence of (T,G)-A--L antibodies induced by the first

immunization. This is because immune cells from a high responder strain transferred into irradiated syngeneic recipients containing (T,G)-A--L antibodies fail to respond to (T,G)-A--L immunization.

Functions usually attributed to T cells are defective in low responder strains. Reactivity can be restored with high responder T cells. However, Ir-IA region genes may also affect T-B cell cooperation. The Ir-IA gene product may provide the stimulus triggering proliferation and differentiation of B cells towards IgG-secreting cells. (Klein, 1975; Markman and Dickler, 1980).

The differences between responder and non-responder strains are further complicated by the fact that with some antigens the H-2 linked gene seems to be the sole controller of immune responsiveness, responsiveness to other antigens is controlled by a whole series of genes.

D. The Major Histocompatibility Complex of Man

The MHC in man is at present defined by at least four distinct loci, HLA-A, -B, -C, and -D. The first three loci code for allo-antigens readily detectable by serological methods, and were first called serologically defined (SD). The fourth locus, HLA-D, controls the proliferative response observed in *in vitro* mixed lymphocyte culture and has been called lymphocyte defined (LD). (Bain et al, 1964; Dupont et al, 1976).

This distinction, however, has become inexact with the observation that DR antigens are expressed on B cells and can be defined serologically. (Bodmer, 1978). It is not clear whether D and DR represent two genetic loci or one. HLA-A, B, and C molecules are determined by allelic genes at the three loci and are present on most, probably all, nucleated cells. They are composed of one variable glycosylated polypeptide chain of about 43,000 daltons noncovalently linked to a constant beta-2 microglobulin moiety of 11,000 daltons (Cresswell et al, 1973; Peterson et al, 1974; Thorsby, 1979).

The beta-2 microglobulin has primary structural homology with immunoglobulin constant region domains. This supports the notion of common evoluationary origins for the immunoglobulin and histocompatibility systems. (Orr et al, 1979). An 88 residue fragment (ac-2) containing the second disulphide loop of the HLA-B7 heavy chain has statistically significant homologies to immunoglobulin constant domains and to beta-2 microglobulin. Occurrence of an immunoglobulin like region in an MHC product suggests that the humoral and cellular arms of the immune system share a common ancestral development.

The HLA-A, B, and C molecules are highly polymorphic. Many molecules carry both "private" antigenic determinants (unique for a given A, B or C molecule) and "public" determinants (shared between different allelic products). These molecules induce antibody production and cytotoxic cellular responses in allogeneic combinations.

The HLA-D/DR molecules are determined by allelic genes in the D region and have a more restricted tissue distribution. They are present on macrophages, B lymphocytes, endothelial cells, and sperms. (David et al, 1973; Dossetor et al, 1978). Composed of two non-covalently linked glycosylated polypeptides of 28,000 and 33,000 daltons, they are also highly polymorphic and carry "private" and "public" determinants. These molecules induce strong T cell proliferation and antibody production in allogeneic combinations (Bach and Hirschhorn,1964). The T cell activating determinants are called D, while those inducing antibody production are called DR.

Most T cells appear to have receptors for allo and/or self-MHC cell membrane molecules, since a given set of allo-MHC molecules triggers many T cells, and self MHC molecules are involved in triggering T cell immune responses to foreign antigens. (Dossetor et al, 1978; Thorsby, 1979).

The most common method of testing for HLA-A, B and C antigens is a dye exclusion microlymphocytotoxicity assay developed by Mittal et al (1968). A modification of this technique is used to test for HLA-DR antigens. (Bodmer, 1978).

1. Immune response genes and disease susceptibility in humans.

In addition to the HLA-A, B, C and D/DR regions of the MHC,

there are closely linked genes that control the ability of an individual to respond immunologically to many antigens. These genes may be the same as, or closely linked to, a group of genes associated with disease susceptibility. Genes in this linkage group are probably also involved in controlling processes involved in cell interaction. (Bach and Van Rood, 1976).

One of the explanations for the maintenance of the diversity observed in the HLA region is the relationship between HLA and disease. (Bodmer, 1972; Degos et al, 1974; Lilly, 1972). An early study in this area showed an increased frequency of two cross-reacting antigens, HL-A5 of Dausset and 4C of Payne, in Hodgkins disease (Amiel, 1967).

A large number of other diseases have since been associated with increased or decreased frequencies of various HLA antigens. These diseases have been classified by Morris (1974) into (1) lymphomas and leukemias, (2) immunopathic diseases, (3) cancer other than lymphomas, and (4) infectious diseases. (McDevitt and Bodmer, 1974). There is now much data arguing for a selective pressure affecting HLA polymorphism. (Degos et al, 1974).

In the mouse model, H-2 correlates with HLA and Ia molecules are believed to be similar to DR molecules (Thorsby, 1978). Ia molecules expressing different allogenic variants may differ in their capacity to display antigenic determinants in an immunogenic way to T lymphocytes either through differences in capacity to combine with, or be modified by, antigen. This implies that many of the HLA and disease associations could be explained by the HLA molecules participating directly in the immune response.

Heterozygosity at HLA, and a high degree of polymorphism, will be an advantage for the species, since the more allotypic variants there are to select from, the higher is the chance that some members will have the appropriate display of HLA-A, B, C, D, molecules with which to mount an efficient immune response against a given infectious agent. Depending on the environment, different selective pressures would exist in different populations, as found by Bodmer. (1973). (Van Rood et al, 1976).

E. Techniques for Study of In Vitro Generated Antibody Formation With Mice

Marbrook (1967) described a technique for primary immunization of cultures of mice spleen cells. Spleen cells from normal, immunized mice are grown on dialysis membranes above a reservoir of medium together with the desired antigen.

Using this method, the cells have close contact with each other, and nutrient supply for cell growth is maintained. Antibody producing cells appear in significant numbers in the cultures within three to five days.

The Jerne and Nordin (1963) assay to measure plaque formation in agar by single antibody-producing cells was used by Marbrook. Distinct plaques, each of which is due to the release of hemolysin by a single antibody forming cell, are revealed by complement after incubation in an agar layer of a mixture of SRBC and lymphoid cells immunized *in vivo*, or *in vitro*, with SRBC. (Jerne et al, 1974).

Incubation of the cultured cell suspension at $37^{o}C$ in the presence of puromycin and cycloheximide (10 µg/ml) decreased the number and size of the plaques. Although the synthesis of antibody must be completely inhibited, the cells can continue for some hours to secrete the antibody synthesized prior to exposure to these drugs. Incubation of cell suspensions for two hours in the presence of 8 µg/ml of Actinomycin D (which inhibits RNA synthesis) has very little effect on plaque formation. It would appear that the messenger RNA responsible for RNA synthesis is long lived (Jerne et al, 1974).

The direct technique yields plaques from cells that produce IgM hemolytic antibody. This is based on the greater efficiency of IgM

in fixing complement, and the rise in direct plaques to a peak on day four following injection of a large dose of SRBC is paralleled by a rise in the titre of specific IgM in the serum. (Jerne et al, 1974).

Antibody directed against IgG can inhibit plaque formation by precipitating the antibody released by potential plaque forming cells (PFC) (Chou et al, 1967). IgG can also result in the appearance of new plaques. This increase in plaque number after treatment with anti-IgG antibody has been attributed to some cells forming IgG of low hemolytic capacity. These two actions of IgG antibody are dependent on the concentration of the antibody added to cultures of sensitized lymphocytes. Therefore, although the IgM plaque numbers may be read quite precisely from a direct plaque forming cell assay, IgG plaque numbers may be calculated only after dilution studies of the anti-IgG serum to be added to the assay. (Chou et al, 1967).

Mishell and Dutton (1967) describe another technique for <u>in vitro</u> stimulation of spleen cells from normal mice. The critical conditions are low oxygen tension, gentle agitation of the cultures, the inclusion of fetal calf serum (FCS), adequate spleen cell density, and daily feeding of the cultures with a nutritional mixture.

Cunningham and Szenberg (1968) developed a plaque assay using two glass slides put together with two sided tape at both ends and in the middle. Aliquots of lymphocyte suspension, SRBC and complement are mixed in microtitre tray wells. The mixture is slid between the two slides by pasteur pipette. The chambers are sealed with heated paraffin and incubated for 30-60 mins. This technique is simpler to perform and gives more accurate readings than the agar techniques as the plaques are in a monolayer between the two slides.

F. Results From Early Murine System Experiments

Early studies with murine lymphocytes by McDevitt and Sela (1965) showed that interstrain variation in the response to SRBC was greater than the variation within a strain of mice. These authors measured antigen binding to the mouse antiserum. The genetic origin of this difference was confirmed by backcross studies.

Experiments with (T,G)-A--L showed the genetic control of antibody production against a relatively well defined antigen. Intermediate hybrid responses suggested a gene dose effect in the heterozygote. It is important to note that responses to these polypeptide antigens are observed in inbred mice only. They are not immunogenic in random bred Swiss mice of various H-2 specificities. (Benaceraff and McDevitt, 1972). This may be due to differences in non H-2 genes.

Lonai and McDevitt (1974) showed that Ir-IA is a dominant autosomal gene(s) which regulates the specific antibody response to several antigens, including three branched multichain polypeptide antigens, (T,G)-A--L, (H,G)-A--L, and (Phe,G)-A--L. This gene is located within the H-2 region between the K end and another immune response gene, Ir-IB. Adoptive transfer experiments showed that Ir-IA is expressed on lymphoid cells and that Ir-IA regulated immune responsiveness is transferrable with lymphocytes from high responder animals into irradiated low-responder animals.

Lymphoid stem cells from responder mice mature normally under the influence of a thymus from a non-responder strain. (Tyan et al, 1969). The specificity characteristics of the Ir-IA gene(s) were shown to be expressed on thumus derived cells.

Responsiveness to another terpolymer containing L-glutamic acid,

L-alanine, and 10% L-tyrosine (GAT10) was found to be controlled in inbred mice by a dominant gene distinct from those previously identified at the Ir-IA locus (Kantor et al, 1963). In contrast to the quantitative differences in antibody response to the branched copolymers, this gene apparently determines responsiveness to GAT10 in an all or none fashion.

Smith et al (1977) showed that two loci, one linked to H-2 and the other linked to the immunoglobulin heavy chain allotype locus (Igl) control the quantitative IgM response to (T,G)-A--L. High response is dominant over low response. Responders may be either heterozygous or homozygous. The progeny of non responders are all non-responders, implying that the non responders are homozygous for this trait. (Pinchuck and Maurer, 1965).

Immune responsiveness to (T-A-G-GLy)n in mice is under the control of at least two separate genes (Merryman et al, 1977). One gene controls the ability to respond and maps to the IA subregion. A non H-2 linked locus is responsible for the magnitude of the response.

Sant'Anna et al (1979) showed that the primary response to f and s antigens of Salmonella Typhimurium is also likely to be under polygenic control.

Quantitative regulation of the response to sheep erythrocytes is now known to be under the control of 7-13 independently segregating loci. (Silver, 1979). Control of the proportions of IgM and IgG produced in response to SRBC is attributable to non H-2 linked genes. These studies indicated that, at least <u>in vivo</u>, genetic control is exerted at various stages in the immune response to SRBC, from the

processing of antigen to immunoglobulin production.

G. Results From Early Work With Human Lymphocytes

1. Tonsillar lymphocytes.

Several early studies of human lymphocyte responsiveness in vitro made use of tonsillar cells. B and T cells are in almost equal proportion in this population. In peripheral blood, lymphocytes are approximately 80% T cells. Despite this difference, important information was gained and several techniques developed which have been used in work with peripheral blood.

For example, Watanabe et al (1974) used Marbrook's technique and Keyhole limpet hemocyanin (KLH) coupled with dinitrophenol (DNP) as antigen in cultures of tonsillar lymphocytes. They observed little response from T or B cells cultured alone. An optimal ratio of T to B cells was observed for each person studied. Depletion of glass adherent cells resulted in elimination of the anti-hapten response. There was a requirement for macrophages even in the presence of 2-mercaptoethanol. The peak response was observed on day 5 or day 6, using Jerne's plaque assay.

Using the Mishell-Dutton system, Luzzati et al (1976) found that for human lymphocytes to respond by making antibody to RBC, 2-mercaptoethanol was required. The peak response was on day 8, measured by a modified Jerne assay. This group was able to increase the response with a specific T cell factor obtained by immunization of mice. 10^8 murine thymocytes were injected into irradiated, (750R) recipients together with .1 ml of 10% SRBC or horse RBC. Seven days later the spleens of the recipients were removed and made into single cell suspensions. The corresponding antigen was added, .1 ml of 1% RBC suspension per ml of culture. They were cultured for six hours

at 37°C. The cells were then spun down, and the cell-free supernatant was the T cell factor.

These experiments suggested that removal of adhering inhibitor cells was necessary, as the PFC response to SRBC was only observed after removal of adherent cells.

Galanaud et al (1977) used this technique with SRBC as antigen and also found that the response was not reproducible without the mouse T cell factor. They therefore used TNP-PAA as antigen, which evokes a T cell independent response. 5-10% of normal test subjects were non-responders in this assay.

2. Peripheral blood lymphocytes.

Peripheral blood lymphocytes stimulated by SRBC have not given reproducible numbers of plaque forming cells (Delfraissy et al, 1978; Fauci and Pratt, 1976). Individual variations are also reported in response to such complex antigens as E. Coli and purified pollen antigens. (Black et al, 1976; Kim et al, 1979). This suggests that the effects of Ir genes may only be observed under extremely limited conditions, such as with structurally simple synthetic polypeptides.

One report of correlation between in vitro immune responsiveness and HLA is the antibody and proliferative response to vaccinia virus. (DeVries et al, 1977). A low in vitro response to vaccinia virus, tested 3-4 weeks and 5-11 weeks after vaccination was associated with HLA-CW3. Responses to phytohemagglutinin (PHA) and other antigens suggested that this response was specific for vaccinia virus. The difference between in vivo and in vitro sensitization, particularly with respect to immune response gene control, is unknown.

The response by PBL to TNP-PAA gives a peak on day 8, with good reproduciblity. (Delfraissy et al, 1977). Adherent cell depletion did not modify the in vitro response, nor did the addition of 2-mercapto-ethanol. Plaques, using the Jerne assay, were inhibited by cyclo-heximide and by anti-human IgM serum, but not by anti-human Fc IgG serum. This indicates that the plaques were of IgM class, and that protein synthesis was required for their development. The removal of T cells from cultures eliminated the response. TNP-PAA stimulated T-enriched cells added to cultures of T-depleted cells did not return a response when added at the time of the PFC assay. Thus, T cells are required at the time of induction. (Galanaud, 1979).

Friedman et al (1976) also studied the response of purified B cells in Mishell-Dutton cultures. They isolated B cells by Sephadex G-200 anti-Fab column chromatography and non specifically activated them with either pokeweed mitogen (PWM) or soluble products of antigen-activated human T-cells, to differentiate into PFC. In contrast, unfractionated peripheral blood lymphocytes, immunoglobulin minus cells isolated from immunosorbant columns, and B cells, enriched by nylon wool adherence, were not triggered to synthesize antibody even in the presence of polyclonal activators. However, B cells isolated by nylon wool adherence and further fractionated by binding and elution from anti-Fab columns were readily triggered by PWM to differentiate into PFC. These results suggest that the interaction of human B cells with anti-Fab columns, presumably with the immunoglobulin receptors, serves as a signal important in the differentiation events leading to PFC activity. The anti-Fab activity alone, although critical, was not sufficient to induce the differentiation of precursor cells

into PFC since subsequent triggering by antigen-activated T-cell supernatants or PWM was required for the development of an optimal PFC response.

PFC responses could be regulated by autologous peripheral immunoglobulin-minus lymphoid cells in vitro. At low concentrations of PWM, immunoglobulin-minus cells consistently augmented the B cell PFC response. At high concentrations of PWM, suppression was observed. Depletion of erythrocyte-rosetting cells from the immunoglobulin-minus population eliminated the subset responsible for augmentation but did not eliminate the suppressor cell activity, suggesting that the T cell population is responsible for helping the B cell response and that some other cell type may be responsible for suppression.

3. Variability in the response to SRBC by one individual.

Lymphocyte proliferation in vitro precedes the development of PFC in response to antigen (Kim et al, 1979). Although the number of PFC observed is not correlated with the amount of thymidine incorporated by lymphocytes in culture. Day to day variation in the numbers of PFC generated by lymphocytes from a single donor may be reduced by using serum supplements from one donor.

Much of the difficulty in measuring the immune response to SRBC in normal individuals has been in this variability of responses observed from one person. (Galanaud et al, 1977; Mills and Paetkau, unpublished data).

Published explanations for day to day variation are,

1. Variations in the T-B cell ratio (Watanabe et al, 1974). Each person tested had a peak response at a different proportion of T to B cells. The variation of this optimal

ratio from experiment to experiment was not studied. Also, the number of platelets, polymorphonuclear cells, and monocytes would be expected to have an effect on the immune responsiveness of an individual.

2. A differing sensitivity of B cells to T cell help or suppression. (Friedman et al, 1976). These results suggested that B cells may have a variable sensitivity to suppression, whether that suppression is due to a suppressor factor, a suppressor T cell, or a suppressor macrophage in a particular system is still uncertain.

3. The presence of suppressor cells in various quantities and of various types for example, macrophages, T cells, B cells. (Luzzati et al, 1976; Waldmann and Broder, 1977). These authors suggest that various immunodeficient states arise due to a preponderance of suppressor cells, probably T cells. In culture, the removal of these cells then allows B cells to function normally.

4. Xenogenic antigens cause non-specific stimulation of human B cells, giving low reproducibility in a PFC assay. (Delfraissy et al, 1978).

5. Variations in the SRBC themselves, their sensitivity to lysis and their antigenicity. (Cunningham and Pilarski, 1974).

6. Complexity due to the polygenic control of the immune response to complex antigens. (Galanaud et al, 1977; Schwartz et al, 1978)., and

7. The natural capacity of an individual's lymphocytes

to proliferate and produce antibody may be affected by infection, or any simultaneous response that they may be making. (Dupont et al, 1976; Ishizaka, 1976). Antigenic competition or cross reactivity of determinants might increase or decrease the response to a particular antigen on some occasions.

H. Development of Suppressor Cell Analysis by PFC Responses With Mice.

SRBC-specific suppressor T cells may be induced in Mishell-Dutton cultures and measured by the Cunningham assay. (Eardley et al, 1976). Varying amounts of antigen in a primary culture are used to study the induction of suppressor cell function as measured in a secondary culture. The production of suppressor cells is favored by increasing the antigen dose in the initial culture but can also be produced by transferring more cells when lower doses of antigen are used.

Transfer of small numbers of cells, cultured with low doses of antigen, led to a specific helper effect. Transfer of large numbers of educated cells led to nonspecific suppression; responses to SRBC and HRBC being decreased, whether or not SRBC or HRBC were present in the educational culture.

Suppression can be induced by the effluent cells from nylon wool columns which do not make detectable plaques. (Eardley et al, 1976).

The suppressor cells are radiation sensitive and must be able to synthesize protein to suppress. Pactamycin, a protein synthesis inhibitor, which binds to ribosomes, was able to abolish the suppressive potential of educated cells. It took two to three days of education to reach the maximum suppressive efficiency. The cells would not suppress cultures if added two to three days after culture initiation.

Kontiainen et al (1978) induced suppressor cell function in vitro in very small numbers, with 10^3 cells, often completely suppressing a primary immune response. Culturing cells for 24 hours with antigen yielded supernates which mediated specific suppression. A metabolic

inhibitor, sodium azide, abolished the release of suppressor factor from suppressor cells. Once helper factor had been released, suppressor factor was inert, as in the previous experiments by Eardly and Gershon (1976). Thus, the target of suppressor factor is the helper pathway and not the B cell or the macrophage.

The anti-erythrocyte autoantibody response in mice injected with rat erythrocytes is regulated by suppressor cells. (Naysmith et al, 1980). The autoantibody response was distinguished from the anti-rat response by being more radiosensitive. Lymphoid cells from mice given rat erythrocytes transferred to normal syngeneic recipients suppressed autoantibody production in the recipients. The degree of suppression was related to the number of cells transferred and to their time of injection relative to the injection of rat erythrocytes. The induction of autoantibody and the generation of suppressor cells in donor animals was unaffected by adult thymectomy. The authors suggest that T cells can specifically inhibit the response of autoreactive B cells, although non-T cells can also suppress their response. How the formation of autoantibody is aided by the injection of rat erythrocytes in this system is being investigated.

Several immunosuppressive drugs have been studied in the mouse system, both <u>in vitro</u> and <u>in vivo</u>. For example, Braciale and Parish (1980) found suppressor lymphocytes in spleens of mice 5-14 days after treatment with high doses of cyclophosphamide. These spleen cells can suppress the <u>in vitro</u> primary response to the soluble hapten-protein conjugate dinitrophenylated monomeric flagellin or HRBC when added before the second day of culture. This suggests that the cyclophosphamide induced suppressors act late in the <u>in vitro</u>

antibody response, possibly by prematurely shutting off antibody synthesis by B cells. They appear to be T cells, being sensitive to treatment with anti-thy 1.2.

I. Development of Suppressor Cell Analysis by PFC Responses With Humans

Using the culture and assay system of Gelfand, (Shore et al, 1978), it was noted that at high antigen concentrations PFC responses were suppressed and it seemed that this effect might be mediated in part by antigen-stimulated T-lymphocytes.

They studied the functional activity of purified T cells which had been pre-incubated or "primed" for 24 hours in the presence of a high concentration of antigen. These cells contained antigen-specific suppressor cells which interfered with the induction of PFC in autologous target PFC cultures. These suppressor cells and their precursors could be isolated in the theophylline-sensitive (T-sens) lymphocyte fraction. Addition of similarly primed theophylline resistant (T-resis) cells resulted in a marked enhancement of PFC generation. The majority of T-sens cells carry receptors for the Fc portion of IgG. IgM receptors were found on T-resis cells. Using IgM or IgG coated ox red blood cells in a rosette depletion procedure, the T-mu and T-gamma subsets contained in the primed, purified T cell preparation were isolated and their activity assayed. Helper cell activity seemed to be restricted to the T-mu enriched subpopulations whereas suppressor cells were found in the T-gamma enriched preparations.

Uytde Haag et al (1978) using Gelfand's technique, also noted suppression in cultures stimulated with a high dose of antigen. They set out to assess two possibilities, that (1) a suppressor cell was activated in these cultures, or (2) B cell inactivation occurred at high antigen concentrations, without any interference from T cells.

PBL were incubated for 24 hours with high doses of antigen. These cells were then added to an equal number of autologous PBL and re-cultured in the presence of antigen. An average of 80% suppression was observed, relative to cultures containing only fresh PBL.

Some PBL were depleted of adherent cells and separated into T and non-T cells. The isolated non-T cells were supplemented with 5% adherent cells and incubated with a high concentration of antigen for 24 hours. The cells of these two populations were then added to normal PBL primed with an optimal dose of antigen 24 hours earlier. These conditions were optimal for the generation of a suppressive effect. Non-T cells were not able to suppress PFC responses in vitro.

This group also studied the supernatants of antigen-specific T suppressor cells, designated TSF24. (Uytde Haag et al, 1979). T lymphocytes were cultured in microtitre plates with SRBC at a ratio of 1 PBL:10 SRBC. After 24 hours, the supernatants were collected and freed of cells by centrifugation. The dose dependency of the effect of TSF24 varies with each donor and protein synthesis is required for its production.

These supernatants (a) can suppress an antibody response of autologous but not allogeneic lymphocytes to the inducing antigen, (b) are antigen specific in their effect, and (c) are produced by radiosensitive T cells. The target of the factor is a radiosensitive T cell. This indicates that in the generation of T effector and suppressor cells in vitro, T-T interactions occur and, in addition, that cell free factors may be involved in these interactions.

Up to 75% of T lymphocytes in human peripheral blood have receptors

for the Fc fragment of IgM (Tm), and a smaller number, less than 20% have receptors for IgG (TG), and the remainder lack detectable receptors for IgM or IgG. (Moretta et al, 1977). Since these two types of receptors are normally present on distinct subpopulations of T cells, it is possible to fractionate T cells with Fc receptors by their capacity to bind IgG or IgM immune complexes and subsequently to analyze their behavior in in vitro assays. T-mu and T-gamma cells respond similarly to the T cell mitogen CON A but differently to PHA, indicating that, in the human, as in the mouse, subpopulations of T lymphocytes can be distinguished on the basis of differential mitogen responsiveness.

These authors found that the helper activity was confined to the T-mu populations. T-gamma cells do not help the induction of either proliferation or differentiation of B cells. After interaction with IgG immune complexes during isolation, T-gamma cells suppress the generation of plasma cells when added to helper T cells and B cells together with PWM.

It has been reported that the generation of a T-suppressor cell effect requires mitosis, or another irradiation sensitive, mitomycin C sensitive process. Fauci et al (1978) found that a subpopulation of suppressor cells in some individuals are even more sensitive to irradiation than are B cells, resulting in the occasional enhancement of responses seen at doses of irradiation lower than those which directly suppress B cell function.

Suppressor cells to the induction of B lymphocytes to differentiate into plasma cells in response to PWM are radiosensitive. (Lydyard and Hayward, 1979). At least two cell types may mediate CON-A induced suppression; one which suppresses directly and is radiosensitive

and the other which is radioresistant and stimulates suppressor precursors in a target population of T cells. These authors report an enhanced proliferative response to CON A in normal individual's PBL pre-cultured for 24 hours in vitro, possibly due to the short in vitro survival of the suppressor precursor. These primary incubations were performed in RPMI plus FCS.

Treatment of human PBL at submitogenic concentrations of sodium periodate completely inhibits their ability to mount a primary in vitro antibody response to TNP-PAA. (Galanaud et al, 1980) Transfer experiments showed that submitogenic doses induce suppressor cells to the in vitro antibody response. The generation of these suppressor cells is decreased but not abolished by mitomycin treatment whereas that of CON-A induced suppressors is prevented. This suppression is present in the T-enriched fraction. The two types of suppressor cells appear to affect the in vitro B cell response differently (Galanaud et al, 1979), both because cell proliferation is required for the expression of CON-A stimulated suppressor cells and because these T cells will suppress equally well when added on day 2 or day 0 of the responding culture.

A summary of these experiments is presented in Table 1.

Table 1. Summary of Suppressor Cell References

Reference	Type of cell	Method of Induction of Suppressor Cell	Technique for Demonstration of Suppressive Effect	Modifying Factors
Shore et al, 1978	T-gamma	pre-incubation with high dose antigen	PFC assay	effect observed by pre-treatment of T cells with theophylline
Uytde Haag et al, 1978; Uytde Haag et al, 1979	T cell, which is radio-sensitive	pre-incubation with high dose antigen	PFC assay	supernatants of the primary cultures suppress autologous secondary cultures
Lydyard and Hayward, 1979	T cell-radio-sensitive T cell-radio resistant	treatment with CON-A	^{3}H-thymidine incorporation	dose of irradiation
Galanaud et al, 1979	T cell	treatment with sodium periodate	PFC assay	mitomycin-C treatment

II. MATERIALS AND METHODS

Plaque forming cell assays were performed using SRBC antigen to stimulate PBL, T, or B cells in culture. The technique was standardized with respect to antigen dose, lymphocyte dose, kinetics, and culture volume.

The responses of freshly prepared PBL were compared to those of freshly reclaimed (from storage at -70°C) PBL. The effect of adding PBL, T, B, or adherent cell populations pre-incubated with or without antigen to autologous cultures was studied.

A. Preparation of Peripheral Blood Leukocytes (PBL)

The method of Boyum (1968) with modifications, was used to prepare mononuclear cells from peripheral blood. Seven ml tubes of heparinized venous blood were collected and centrifuged at 1500 rpm (500 x g) for seven mins. About 1 ml of buffy coat was collected from each tube and mixed with equal volumes of medium RPMI-1640. About 5 mls of this buffy coat suspension were layered over 5 mls of Ficoll-Hypaque (F-H) (sp. gr. 1.077) in 17 x 100 mm clear falcon plastic tubes and centrifuged at 2000 rpm (800 x g) for 20 mins. Leukocytes at the interface were collected with a pasteur pipette and transferred into another plastic tube, washed three times and resuspended in RPMI. This method regularly gave greater than 95% mononuclear cells. Viability was estimated at over 98% by dye exclusion with trypan blue (0.02% mixed in 1:1 ratio with a cell suspension).

B. Separation of Different Subpopulations of PBL

PBL were prepared from heparinized blood as described above, subjected to rosetting with SRBC followed by centrifugation on F-H to separate the rosetted T cells. In some experiments PBL were

layered in a plastic petri dish to allow monocytes to adhere to the plastic surface. Adherent cells (monocytes) were removed by scraping with a rubber policeman.

In experiments involving pre-incubation of T and B cells, nylon wool lymphocyte separation was performed to avoid exposure of the lymphocytes to sheep erythrocytes before culturing.

1. Preparation of T lymphocytes by sheep erythrocyte (SRBC) - rosetting

The method of Pellegrino et al (1975) was used with the modifications. This technique requires the preparation of amino-ethylisothiouronium hydrobromide (AET) treated SRBC and SRBC absorbed fetal calf serum (FCS).

a. Preparation of aminoethylisothiouronium hydrobromide (AET) treated SRBC

SRBC collected in Alsever's solution and stored less than two weeks in the cold were used throughout this project. Of these, 0.2 ml were packed and washed three times with RPMI-1640. This was added to 0.8 ml of 8% AET (Sigma, St. Louis, Mo.), prepared in distilled water and adjusted to pH 8.0 with 10 N NaOH. This mixture was incubated at 37^{o}C for 20 mins. in a shaking water bath. These cells were then washed three times with RPMI-1640. 0.2 ml of the SRBC were resuspended in 8.0 ml of RPMI-1640 and 2.0 ml of FCS which had been absorbed with SRBC.

b. Preparation of SRBC absorbed FCS

FCS was absorbed with SRBC for use in rosetting to remove naturally occurring anti-SRBC antibodies. (Fauci and Pratt, 1976).

FCS was first decomplemented at 56^{o}C for 30 mins. and then

mixed with SRBC at a ratio of 2:1 (33% suspension). The mixture was incubated at room temperature overnight. The tubes were spun at 1500 rpm (500 x g) for 10 mins. The serum was separated and stored at -70°C for future use.

c. E-rosetting technique and separation of rosetted cells from non-rosetted cells

PBL were prepared as described above and adjusted to a concentration of 5.0×10^6/ml. Equal volumes of these PBL and AET-treated SRBC were mixed. 5.0 ml of this suspension were layered onto 5.0 ml of F-H and the tubes incubated at 4°C for 30 mins. The tubes were then centrifuged at 2000 rmp (800 x g) for 12 mins. at 4°C. Rosetted T lymphocytes and free SRBC settled to the bottom of the tube while non-rosetted cells containing B cells and monocytes remained at the interphase. The latter were transferred into another test tube by pasteur pipette. T cells in the pellet were recovered by hypotonic lysis of the SRBC. 9.0 ml of 0.84% NH_4CL in distilled water were added to the tubes with 1.0 ml RPMI-1640. The tubes were inverted once and then spun at 1500 rpm (500 x g) for 7 mins. These T cells were then washed three times with RPMI-1640. Purity of the preparations was checked by rerosetting with AET-treated SRBC.

2. Preparation of Monocytes

PBL were prepared as described above and incubated, 10×10^6 cells in 15 ml RPMI-1640, on Falcon plastic plates at 37°C for 45 mins. in 10% human AB serum. Non-adherent cells were removed by pasteur pipette, washed by spinning at 1500 rpm (500 x g) for 7 mins. and resuspended in RPMI-1640. The plates were then scraped with a rubber policeman and washed 2-3 times with about 10 ml RPMI-1640 to remove

adherent cells. The adherent cells were washed once and resuspended in RPMI-1640.

3. Nylon wool separation of T and B lymphocytes

PBL were prepared as described above, and adjusted to a concentration of 10 x 10^6/ml. 10 cc syringes were loaded with 0.6 g nylon wool/10 x 10^6 cells. The columns were sterilized with boiling water and then by liberal washing with cold RPMI-1640. The cells were then added to the column and topped with 1 ml RPMI-1640. The columns were incubated for 45 mins. at 37°C. The effluent, T cell rich, population was then recovered by washing of the column with 30 ml of RPMI-1640. The nylon wool was then removed to a petri dish and mashed gently with the syringe top to remove the adhering B cell population, into 15 ml of RPMI-1640.

C. Tests for Checking the Purity of Subpopulations of PBL

1. Rerosetting with AET-treated SRBC

Cells were suspended in RPMI-1640 at 3 x 10^6/ml and 0.25 ml were mixed with 0.25 ml AET-treated SRBC. To this 0.5 ml of SRBC-absorbed FCS were added and the mixture centrifuged at 1000 rpm (200 x g) for 5 min. and left at 4°C for 1 hour. After gentle resuspension the percentage of rosetting cells was checked.

2. Percent killing with anti-B-cell serum

The standard complement dependent cytotoxicity test (Mittal et al, 1968) was adapted with the use of anti-B-cell serum (mouse Ia alloantiserum, Cedarlane, Hornby, Ontario). The percent killing of all T-cell and B-cell preparations were recorded.

D. Freezing and Reclaiming of Lymphocytes

In experiments where reclaimed cells were used, PBL were first prepared as described above. Cells were frozen at a final concentration of 10×10^6/ml in 15% AB serum.

Cell suspensions were frozen by the slow addition of 15% AB serum in RPMI-1640 with 20% dimethyl sulfoxide (DMSO) (Fisher Scientific, Fair Lawn, New Jersey); an equal volume to the cell suspension (at a concentration of 20×10^6 PBL/ml). Cells were stored in NUNC freezing ampules at -70°C. (Inter Med, Roskilde, Denmark)

E. In Vitro Sensitization of PBL to Sheep Erythrocytes

The technique of Dosch and Gelfand (1977) was modified and standardized for use in this project.

The special considerations were; the culture medium, the serum pool preparation, and the sheep erythrocytes.

1. The culture medium

RPMI-1640 was supplemented with 1% trypticase soy broth (BBL, Cockeysville, Maryland); antibiotics, 100 U/ml Penicillin, 100 U/ml Streptomycin, and 25 µg/ml Fungizone (Gibco, Grand Island, New York); 1% 20 mM L-Glutamine (Gibco, Grand Island, New York); and 10% AB serum.

2. Human AB serum pool

Serum pools contained AB serum from at least two donors. Each was first decomplemented by heating in a 56° water bath for 30 mins. The pooled serum was absorbed with 10% SRBC at 4°C for 2 hours to remove naturally occurring anti-SRBC antibodies.

3. Sheep erythrocyte antigen

SRBC were washed three times in RPMI-1640 before use. Cultures

were stimulated with various numbers of SRBC added in 50 λ of RPMI-1640, regardless of the total number to be added. During washing of the SRBC, the buffy coat was removed to avoid the production of autohemolytic antibody forming cells (Jerne et al, 1974).

4. Culture protocol

Lymphocytes and lymphocyte subpopulations were prepared as described above. After washing, they were cultured in culture medium, with or without antigen, at 37°C in 5% CO_2.

The effects of culture period, and numbers of cells, both lymphocytes and SRBC per culture were investigated to establish optimal conditions. Except in the experiments where the volume varied, as described below, the cells were cultured in 10 ml of medium.

Cultures were performed in 17 x 100 mm clear Falcon plastic tubes, or, when the volume exceeded 10 ml, Corning polystyrene tissue culture flasks were used (Corning Glass Works, New York).

F. Plaque Forming Cell Assay

Cells were recovered from cultures by centrifuging at 1500 rpm (500 x g) for 5 mins. The cultures were washed once in RPMI-1640 and resuspended at 2 x 10^6/ml viable cells, as measured by dye exclusion with trypan blue.

The plaque assay was performed in microtest II plates (Falcon plastics #3040) with flat bottom wells.

Poly-L-lysine (PLL) (Sigma, St. Louis, Mo.) was added to each well, 50 λ , (50-100 μg/ml in distilled water) so that its positive charge would allow adhesion of a monolayer of sheep erythrocytes. Excess PLL was removed by rinsing with saline, after 30 mins. incubation of the plates at 37°C.

100 λ of 1-2% SRBC in RPMI-1640 were then added to each well. The plates were then incubated at 37° for 30-60 mins. Excess red cells were removed with saline. 50 λ of RPMI-1640 were added to each well.

25 λ of the resuspended lymphocytes (50,000 cells) were then added, in triplicates, to the plates.

Guinea pig complement (Gibco, Grand Island, New York) was diluted 1:20 with RPMI-1640 and absorbed with SRBC in a 2:1 ratio at 4°C for 1 hour to remove naturally occurring anti-sheep antibodies. 20 λ of complement was added to each of the test wells.

Plates were incubated at 37°C for 1 hour and read on an inverted microscope (Leitz, Wetzlar, Germany).

G. Calculation of Results

Plaque counts from each well were recorded and multiplied by 20 to obtain a PFC count per 10^6 viable cells recovered. The average count and standard deviation for the three replicate wells from each culture and control were recorded.

H. Controls

In every experiment negative control wells with no complement added were assayed. For each individual a "no antigen" control culture was set up.

A zero PFC count for any individual was accepted only if the viability of the culture exceeded 85% and if another individual in the same experiment gave a positive result.

I. Comparison of Responses from Fresh and Reclaimed Cells

An individual's lymphocytes from one bleed were divided into two aliquots, one to be frozen as described above, the other was left

in culture medium at room temperature until the frozen sample was reclaimed. After 2-3 hours at -70°C, the frozen aliquot was reclaimed and both were set up in culture using the same protocol.

PFC counts were compared when the two types of cells were cultured separately, and when added together in varying proportions.

J. Addition of Pre-incubated Lymphocytes to Autologous Cultures

In all these experiments, cells were obtained from one bleed and stored at -70°C as described above.

Lymphocytes, B cells, T cells, and adherent cells were pre-incubated for 1,3, or 6 days in various media and then added to autologous PBL in the standard medium. The pre-incubations, or primary cultures,contained 0, 10^7, 10^8, or 10^9 SRBC and 1.0×10^6 lymphocytes. All target, or secondary, cultures contained 0.5×10^6 freshly reclaimed PBL, 10^8 SRBC, and 0.5×10^6 cells from the primary culture. Control cultures contained 1.0×10^6 freshly reclaimed PBL.

K. Statistical Methods

Tests for significant differences (p values) were performed using the student's t-test. Unless specified otherwise, these tests were unpaired. In all cases all the raw data (each of three triplicates) were used.

Summary of Pre-Incubation Study Protocol

Primary Culture

1, 3 or 6 days

1.0×10^6 PBL, T, B, or adherent cells

+

0, 10^7, 10^8, or 10^9 SRBC

-

Into Two Secondary Cultures

PBL (autologous, 0.5×10^6)	PBL (autologous, 0.5×10^6)
+	+
10^8 SRBC	No Antigen
+	+
0.5×10^6 1^oCells	0.5×10^6 1^o Cells

III. RESULTS

A. Control PFC Results

No PFC were observed in cultures from any individuals unless SRBC were present in the stimulating culture. This lack of background plaques is consistent with work by Ginsburg et al (1978).

B. Purity of Cell Separations

1. T cell enriched

The purity of the T cell enriched fractions was 91 $\pm$ 7.3% by rerosetting. The anti-B cell treatment killed only 4.1 $\pm$ 1.3% of these cell populations. (Table 2)

2. B cell enriched

B cell enriched fractions were 60 $\pm$ 3.5% killed by anti-B cell serum treatment. Only 5.2 $\pm$ 1.1% of these cell preparations rerosetted with AET-SRBC. (Table 2)

3. Monocytes

The purity of adherent cell preparations and of adherent depleted populations was not determined.

C. Kinetics of the PFC Response

To study the kinetics of the PFC response, replicate cultures of varying numbers of lymphocytes and SRBC were harvested from day 1 to day 14. The peak response from all individuals was observed on day 6. This was true regardless of the antigen or lymphocyte dose in culture. Responses were also observed on days 3 through 9. In six experiments where cultures were continued through day 14, no response was observed after day 9. (Figure 1). Complete results are shown in Appendices 1 through 3.

D. Effect of Varying Antigen Dose On the PFC Response

Individuals were tested in cultures containing 10^7, 10^8, or 10^9 SRBC. The peak response was always observed in the culture which contained 10^8 SRBC. Sub-optimal responses were obtained in those

cultures containing 10^7 or 10^9 SRBC. This was true at lymphocyte dosages of 0.25×10^6 (n=17), 0.5×10^6 (n=14), 1.0×10^6 (n=17), 3.0×10^6 (n=11), and 7.0×10^6 (n=8) per culture. (Figure 2). Some individuals gave responses only when cultures contained 10^8 SRBC. Figure 3 emphasizes the point that 10^8 SRBC/culture is the optimum antigen dose at each of the three optimal lymphocyte doses. (0.5×10^6/culture, 1.0×10^6/culture, 3.0×10^6/culture). Complete results are shown in appendices 4 through 11.

E. Effect of Varying Lymphocyte Dose on the PFC Response

Seventeen individuals were tested at five lymphocyte doses; 0.25×10^6/culture, 0.5×10^6/culture, 1.0×10^6/culture, $3.0\ 10^6$ / culture, and 7.0×10^6/culture. These dosages were each tested with 10^7, 10^8, and 10^9 SRBC per culture. The average peak response was at 0.5×10^6 PBL per culture although four individuals gave a higher response at 1.0×10^6 PBL per culture. (Figure 4).

F. Effect of Volume Variation on the PFC Response

To study the effects of nutrient supply and cell crowding the culture volume and cell concentrations were varied. Six individuals were tested at the standard volume of 10 ml, and at two concentrations each; 0.1×10^6 PBL per ml, and 1.0×10^6 PBL per ml. (Figure 5). Three numbers of lymphocytes per culture were studied in each case; 0.5×10^6, 1.0×10^6, and 3.0×10^6. All cultures were stimulated with 10^8 SRBC.

G. Effect of Lymphocyte Treatment with Mitomycin C on the PFC Response

To test for the requirement for DNA synthesis in plaque formation, lymphocytes were treated with mitomycin C before culture.

Treatment consisted of a 30 minute incubation of 5×10^6 PBL

with 25 ug Mitomycin C. The cells were then washed and set up in cultures as usual. Results as shown in Table 3; this treatment significantly lowered ($p < 0.01$) but did not abolish the PFC responses in four individuals.

H. Variation Between Experiments

To check the technical reproducibility of this assay, the responses from four different individuals tested on four different days each were recorded (Table 4). Each culture contained 1.0×10^6 PBL and 10^8 SRBC. The mean responses on each of the four days, and their standard deviations, are compared.

I. Variation Within an Experiment

Within experiment variation was studied by setting up five replicate cultures of 0.5×10^6 PBL and five of 1.0×10^6 PBL from one bleed. Each culture was stimulated with 10^8 SRBC. The mean and standard deviations of six individuals tested in this manner are recorded in Table 5. This shows that there was very low variation within replicates, indicating that this is not the cause of the observed day to day variation from individuals.

J. Day to Day Variation

Day to day variation was studied by testing 20 individuals on four different occasions each. Cultures of 1.0×10^6 PBL were stimulated with 10^8 SRBC. The means and standard deviations for the 20 individuals are recorded in Table 6. Of particular interest are (1) the fairly consistent mean responses and, (2) the wide range of standard deviations, i.e. some people vary much more than others in their day to day ability to form anti-SRBC plaques. The p values for the difference between an individual's high and low PFC responses are all significant. The sources and distribution of day to day variation were not studied; so this variation cannot be further analyzed.

K. Effect of Lymphocyte Proliferation in "Inducing" Cultures on PFC Results

The day to day variability observed might have been due to varying cell proliferation rates. Proliferation indices are compared with lymphocyte dosages (0.5×10^6, 1.0×10^6, 3.0×10^6 and 7.0×10^6 PBL per culture) at 10^8 SRBC per culture (Figure 6) and 10^9 SRBC per culture (Figure 7). Proliferation Index = $\frac{\text{cell count after culture}}{\text{cell count before culture}}$.

L. Effect of Adherent Cell Removal on the PFC Results

The effect of adherent cell removal was studied at 0.5×10^6 PBL per culture and at 1.0×10^6 PBL per culture. All cultures were stimulated with 10^8 SRBC. The adherent cell depleted cultures showed significantly decreased responses, ($p < 0.025$) but their PFC counts were never zero. (Figure 8).

M. Requirement for T and B Lymphocytes

The requirement for T cell help for the B cell function of plaque formation was studied by performing lymphocyte separations. Lymphocytes separated into T and B cell subpopulations by SRBC-rosetting were added together in various proportions, cultured, and tested as usual. All cultures contained a total of 1.0×10^6 cells and were stimulated with 10^8 SRBC (Table 8). Proliferation indices were not correlated with high or low PFC responses. T or B cells alone gave zero PFC; the optimal ratio for anti-SRBC PFC generation was 50% or 80% T cells..

N. Comparison of T/B Cell Separations by SRBC-Rosetting and by Nylon Wool Column Adherence

Since SRBC were the stimulating antigen, the SRBC-rosette method of T cell separation might have had some effect on the results. To

explore this possibility, another method of T cell separation (nylon wool column adherence) was used and the results compared in two experiments. (Table 7). Lymphocytes separated by the two methods were added together in various proportions and tested as usual. All cultures contained 1.0×10^6 cells in total and were stimulated with 10^8 SRBC. The two techniques did not give significantly different results.

O. Studies With Frozen Reclaimed Lymphocytes

As part of the reproducibility studies, the responses from frozen reclaimed PBL were compared with those from fresh lymphocytes. Lymphocytes from one bleed were tested as fresh and as frozen, reclaimed lymphocytes (technique as described in materials and methods). Four individuals were tested in this fashion on four different occasions. All cultures were stimulated with 10^8 SRBC. Three lymphocyte dosages were tested; 0.5×10^6, 1.0×10^6 and 3.0×10^6 PBL per culture. The mean responses from reclaimed lymphocytes were always significantly higher than those from fresh lymphocytes. The standard deviations from experiments with reclaimed lymphocytes were always lower than those from experiments with fresh lymphocytes. (Table 9). Proliferation indices in reclaimed cultures did not correlate with high or low PFC responses and were no less variable than those from cultures of fresh lymphocytes (Figure 9).

A further investigation of twenty-seven individuals comparing PFC responses of fresh and reclaimed lymphocytes showed that at each of three lymphocyte doses per culture, (0.5×10^6, 1.0×10^6 and 3.0×10^6) the responses of reclaimed lymphocytes were always significantly higher. (Figure 10).

Since the freezing procedure involves treatment of lymphocytes

with DMSO, it is possible that the observed differences between the responsiveness of fresh cells and freshly relcaimed lymphocytes are due to the effect of DMSO. To test this possibility, in some experiments (Table 10), cells were treated with DMSO, as described in materials and methods, immediately washed and set up in cultures. Other aliquots from the same bleed were either set up in fresh cultures or frozen, reclaimed, and set up in culture 2 hours later. These results suggest that DMSO treatment alone could conceivably be sufficient to induce the increased response noted above; freezing is not required. Most p values comparing fresh and treated cultures were highly significant (Table 10).

To explain this hypothesis that DMSO treatment removes a suppressor cell or a suppressor cell precursor from the fresh lynphocyte population, fresh and reclaimed lymphocytes were mixed in one preliminary experiment. Cultures were stimulated with 10^8 SRBC and contained 1.0×10^6 PBL (Figure 11a) or 3.0×10^6 PBL (Figure 11b). These experiments showed an increased PFC response as the proportion of fresh lymphocytes was reduced. The same trend was observed in a further series of experiments (Figure 12).

P. Effect of Addition of Pre-incubated Cells to Autologous Cultures on the PFC Response

As we began to explore the activity of suppressor cells in this system, we wondered if pre-incubation of lymphocytes might have the same effect as has been observed in other systems. Pre-incubation activated suppressor cells in humoral systems measuring PFC response to PWM (Lipsky et al, 1978), and to ovalbumin (Shore et al, 1978).

1. Pre-incubation of PBL

Lymphocytes incubated for three days with or without antigen were able to suppress the response of cultures of autologous PBL to SRBC. Pre-incubations (primary cultures) contained 1.0×10^6 PBL. 0.5×10^6 of these

cells were added to 0.5×10^6 freshly reclaimed autologous PBL. These secondary cultures (containing "target" TBL) were stimulated with 10^8 SRBC (Figure 13). The p values, comparing the control cultures to those containing added pre-incubated cells, are: $p < 0.01$ for cells pre-incubated without antigen or with 10^9 SRBC, $p < 0.02$ for cells pre-incubated with 10^7 SRBC and $p < 0.4$ for cells pre-incubated with 10^8 SRBC.

More significant suppression was obtained when the length of the primary culture was six days. All other aspects of the protocol were identical (Figure 14). The p values, comparing the control cultures to those with added pre-incubated cells, are recorded in Figure 14.

2. Pre-incubation of T cells, B cells, and monocytes

To test which type of cells was able to suppress the PFC response, lymphocytes (unseparated), T cells, and B cells were pre-incubated for either one or six days with or without antigen. Nylon wool column separated cells were used and cell numbers were the same as those in the above experiments (Figure 15). B cells which were pre-incubated alone suppressed the target lymphocyte's response to a lesser degree than did PBL; T cells completely suppressed the target lymphocyte response to SRBC. The p values, comparing the control cultures to those with added pre-incubated cells, are recorded in Figure 15 and are all highly significant.

Similar results were obtained when the length of the primary culture was six days. All other aspects of the protocol were identical (Figure 16). The p values, comparing the control cultures to those with added pre-incubated adherent cells, are: $p < 0.05$ for cells pre-incubated without antigen, and $p < 0.01$ for cells pre-incubated with 10^9 SRBC.

3. Quantitation of T suppressor cell activity

Since pre-incubated T cells, 0.5×10^6 per culture were able to

completely suppress the PFC response, a comparison study with fewer added cells was performed. T cells which had been pre-incubated with or without antigen for 24 hours, and T cells which had been freshly reclaimed, were added to autologous, freshly reclaimed PBL (0.5×10^6) and stimulated with 10^8 SRBC (Figure 17).

T cells pre-incubated with 10^9 SRBC suppressed the PFC responses at lower numbers than did those T cells pre-incubated without antigen, most of these comparisons were statistically significant (Table 11). Suppressor indices for these experiments are recorded in Table 11.

Suppressor Index=

$$\left[1 - \frac{\text{Response with pre-incubated T cells added to autologous PBL}}{\text{Response with freshly reclaimed T cells added to autologous PBL}}\right] \times 100$$

The number of cells which had to be added to the autologous PBL to reach 50% suppression of the control response (autologous PBL with freshly reclaimed T cells) is also recorded in Table 11.

4. Varying media in the primary culture

The activation of suppressor cells in primary cultures which lack added antigen but do contain serum has been reported before but never sufficiently explained. (Anaclerio et al, 1979; Burns et al, 1975) The effects of AB serum, autologous serum, or no serum in the primary culture were compared (Table 12).

T cells were isolated, pre-incubated and added to secondary cultures as above. No antigen was present in the primary culture.

Suppression of the autologous PBL response to SRBC, was complete except where the primary culture contained McCoy's medium or RPMI-1640 without serum.

FIGURES

Figure 1. Kinetics of the PFC Response. The mean responses, ± 1 SD, are plotted for six individuals tested on days 1 to 14. All responses before day 3 and after day 8 were zero. All cultures were stimulated with 10^8 SRBC. Cultures stimulated with 10^7 or 10^9 SRBC gave zero PFC. For complete results see appendices 1, 2 and 3.

Figure 1

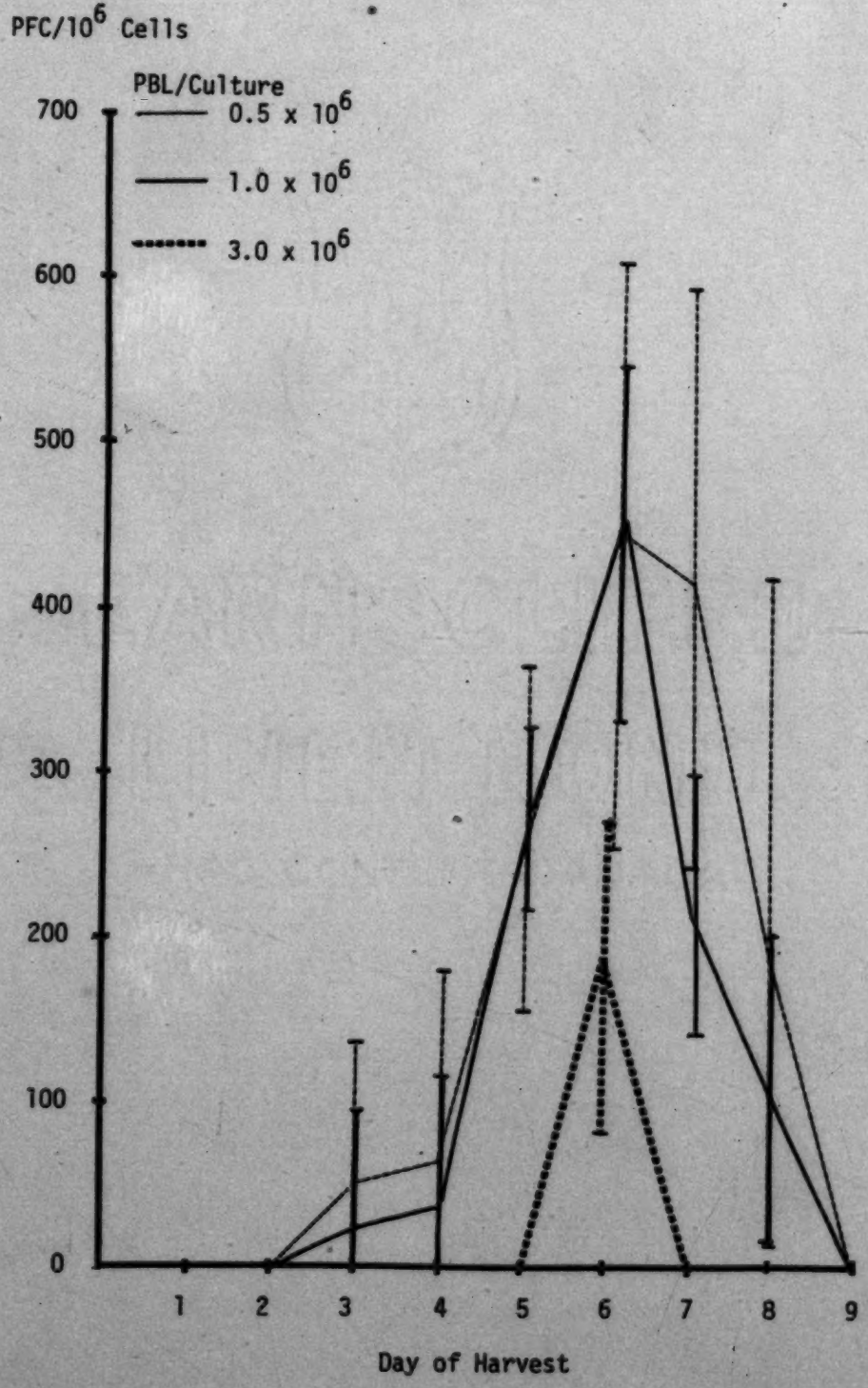

PFC/10^6 Cells
PBL/Culture
0.5 x 10^6
1.0 x 10^6
3.0 x 10^6
700
600
500
400
300
200
100
0
1
2
3
4
5
6
7
8
9
Day of Harvest

Figure 2. Effect of varying antigen dose on the PFC response. The mean responses, ± 1 SD are plotted for individuals tested at three antigen doses. 17 individuals were tested with 0.25×10^6 PBL/culture, 14 individuals were tested with 0.5×10^6 PBL/culture, 17 individuals with 1.0×10^6 PBL/culture, 11 individuals with 3.0×10^6 PBL/culture and 8 individuals with 7.0×10^6 PBL/culture. For complete results see appendices 4, 5, 7, and 9.

Figure 2

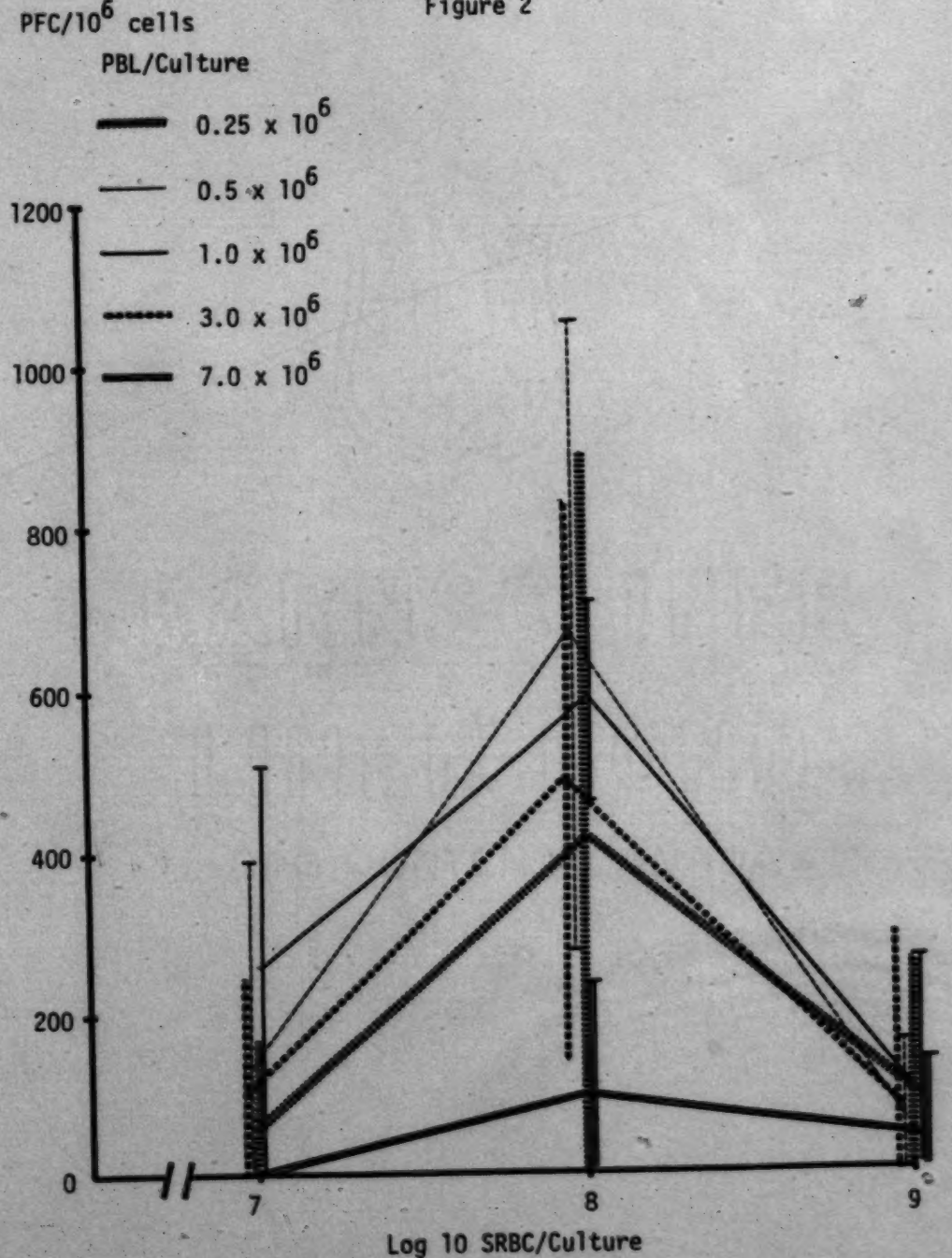

PFC/10^6 cells
PBL/Culture
0.25 x 10^6
0.5 x 10^6
1.0 x 10^6
3.0 x 10^6
7.0 x 10^6
1200
1000
800
600
400
200
0
7
8
9
Log 10 SRBC/Culture

Figure 3. Effect of varying antigen dose on the PFC Response. The mean fraction of the maximum response ± 1 SD, is plotted for three dose response curves in figure 2. For complete results see appendices 6, 8 and 10.

Figure 3

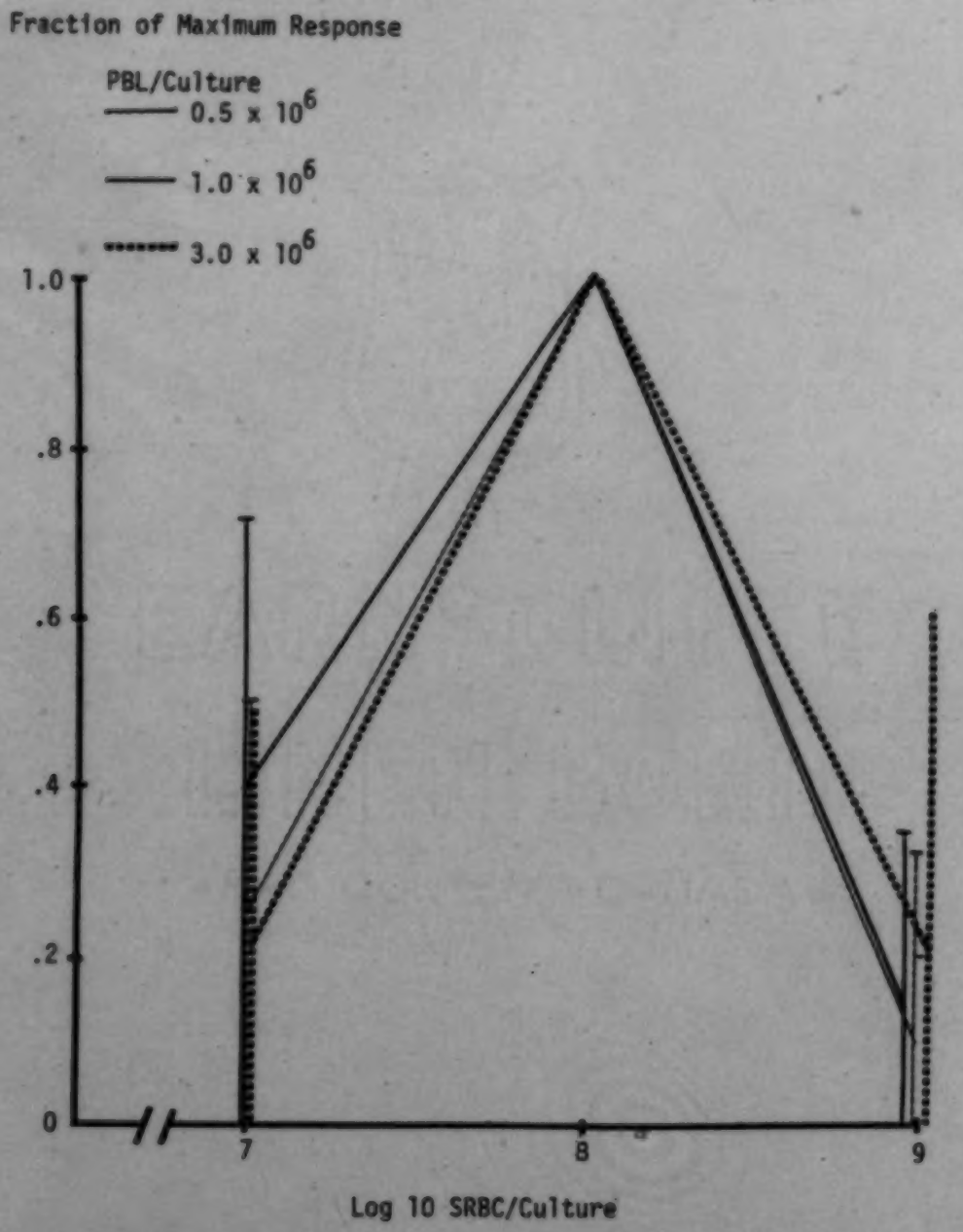

Fraction of Maximum Response
PBL/Culture
0.5 x 10^6
1.0 x 10^6
3.0 x 10^6
1.0
.8
.6
.4
.2
0
7
8
9
Log 10 SRBC/Culture

Figure 4. Effect of varying lymphocyte dose on the PFC response. The mean responses, ± 1 SD are plotted for 11 individuals tested at 5 lymphocyte doses. All cultures containing 7.0×10^6 PBL gave zero PFC.

Figure 4

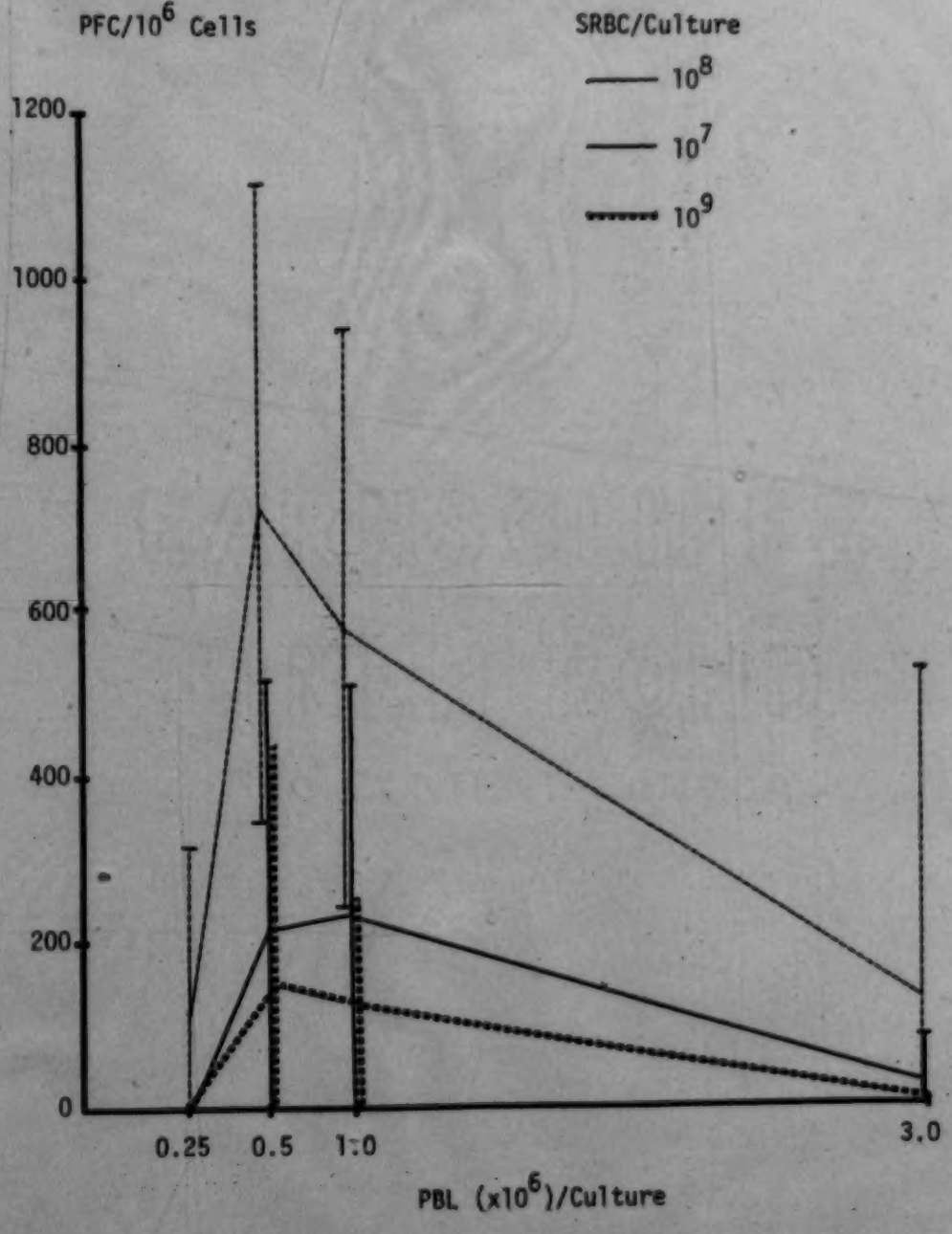

PFC/10^6 Cells
SRBC/Culture
10^8
10^7
10^9
1200
1000
800
600
400
200
0
0.25
0.5
1.0
3.0
PBL (x10^6)/Culture

Figure 5. Effect of volume variation on the PFC response. The mean responses, ± 1 SD, are plotted for 6 individuals tested at two concentrations of PBL/ml. When the volume was held constant at 10 ml, the concentration in each culture was different. All cultures were stimulated with 10^8 SRBC.

Figure 5

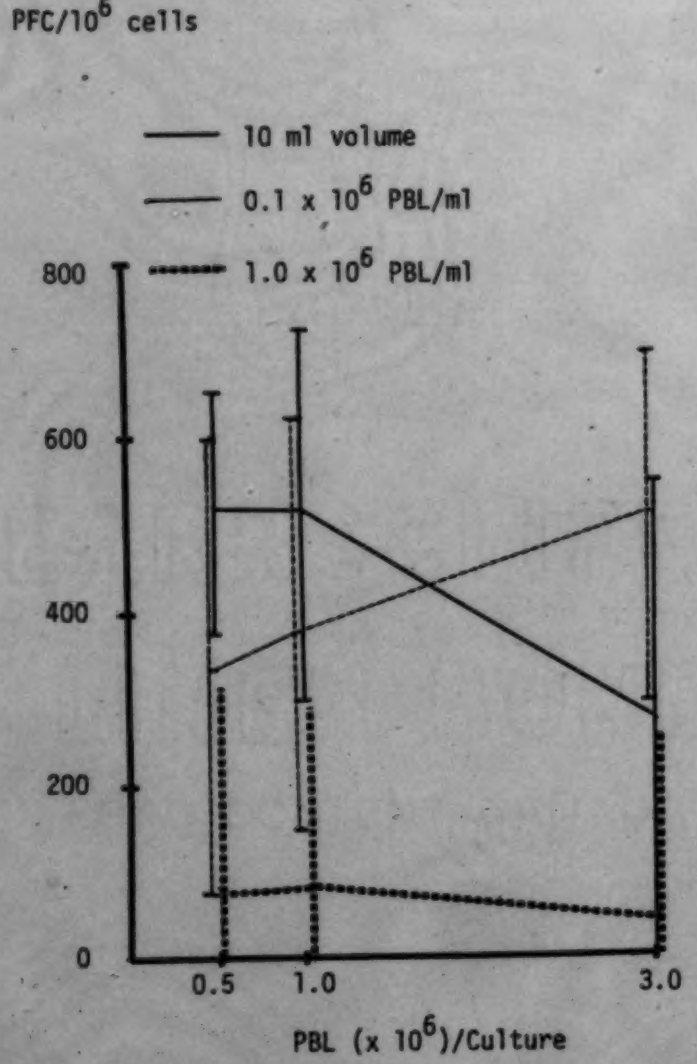

PFC/10^6 cells
10 ml volume
0.1 x 10^6 PBL/ml
1.0 x 10^6 PBL/ml
800
600
400
200
0
0.5
1.0
3.0
PBL (x 10^6)/Culture

Figure 6. Effect of lymphocyte proliferation in "inducing" cultures on PFC results. Proliferation indices are plotted at each of three cell numbers/culture. All cultures were stimulated with 10^8 SRBC. The correlation coefficient for cultures of 0.5×10^6 PBL is 0.69, the other coefficients are less than 0.5. Proliferation Index = Cell count after culture/ cell count before culture.

Figure 6

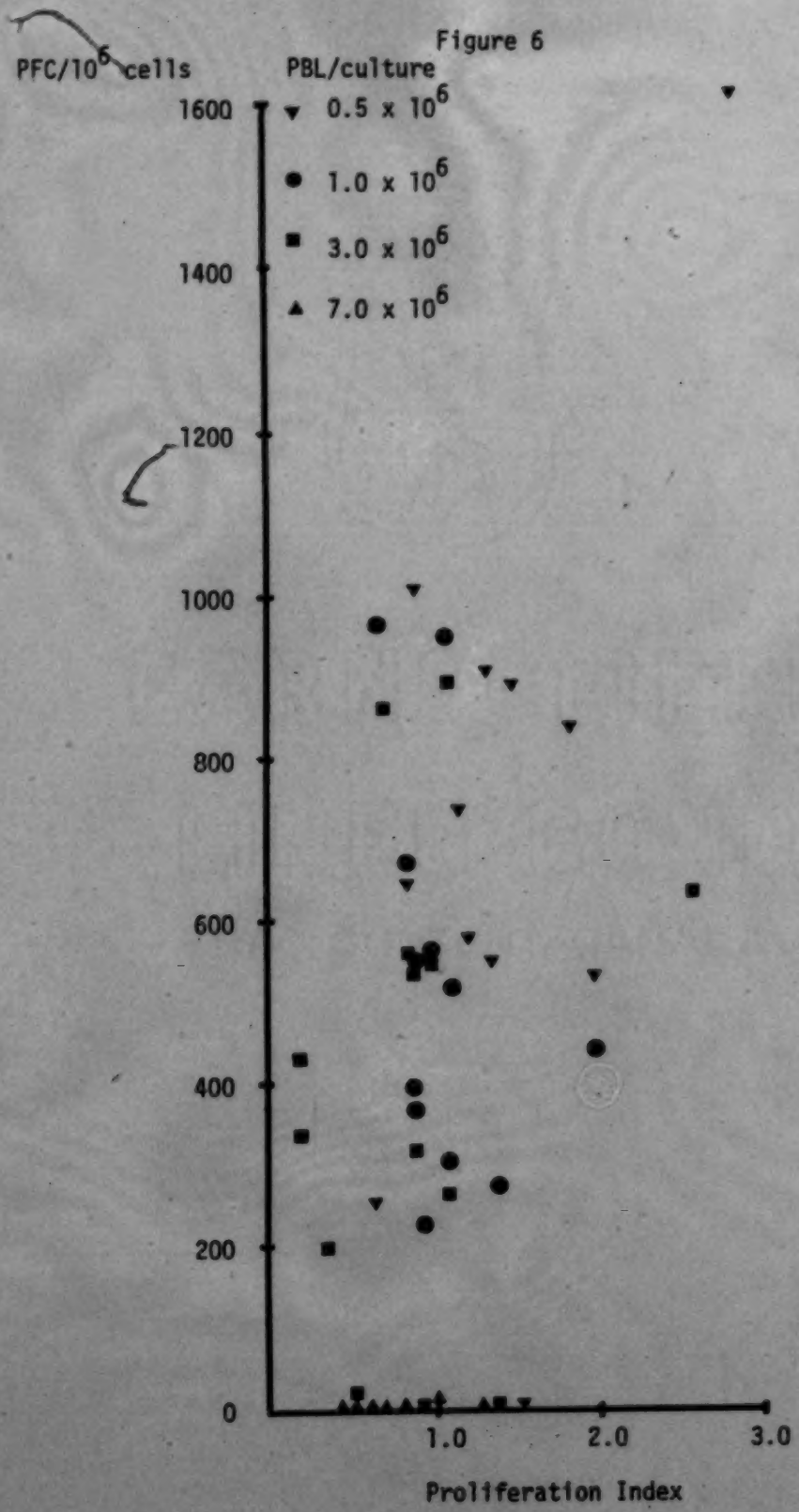

PFC/10^6 cells
PBL/culture
0.5 x 10^6
1.0 x 10^6
3.0 x 10^6
7.0 x 10^6
1600
1400
1200
1000
800
600
400
200
0
1.0
2.0
3.0
Proliferation Index

Figure 7. Effect of lymphocyte proliferation in "inducing" cultures on PFC results. Proliferation indices are plotted at each of three cell numbers/culture. All cultures were stimulated with 10^9 SRBC.

Figure 7

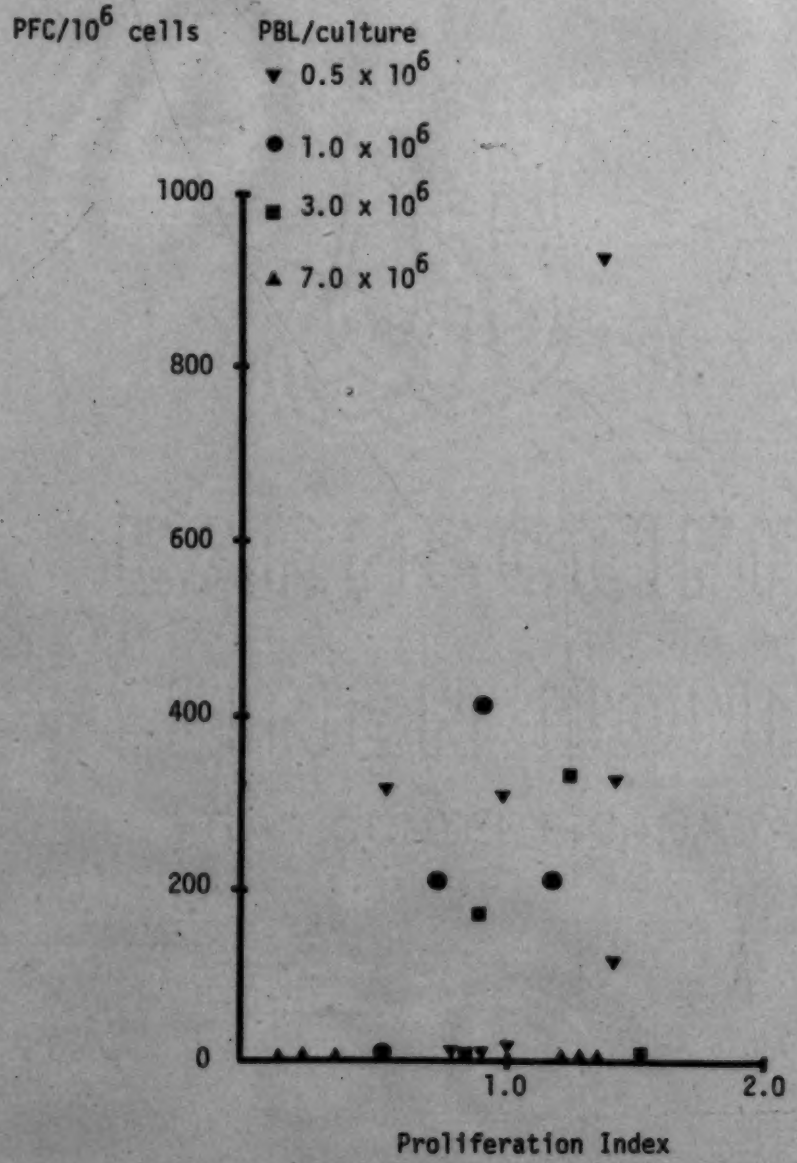

PFC/10^6 cells
PBL/culture
0.5 x 10^6
1.0 x 10^6
3.0 x 10^6
7.0 x 10^6
1000
800
600
400
200
0
1.0
2.0
Proliferation Index

Figure 8. Effect of adherent cell removal on PFC results. PBL were tested with and without adherent cell depletion. All cultures were stimulated with 10^8 SRBC. Mean for control cultures of 0.5×10^6 PBL = 542 ± 143 PFC/10^6 cells. Mean for adherent cell depleted cultures of 0.5×10^6 cells = 506 ± 82 PFC/10^6 cells. This difference is significant, $p < 0.025$, using a paired t-test.

Figure 8

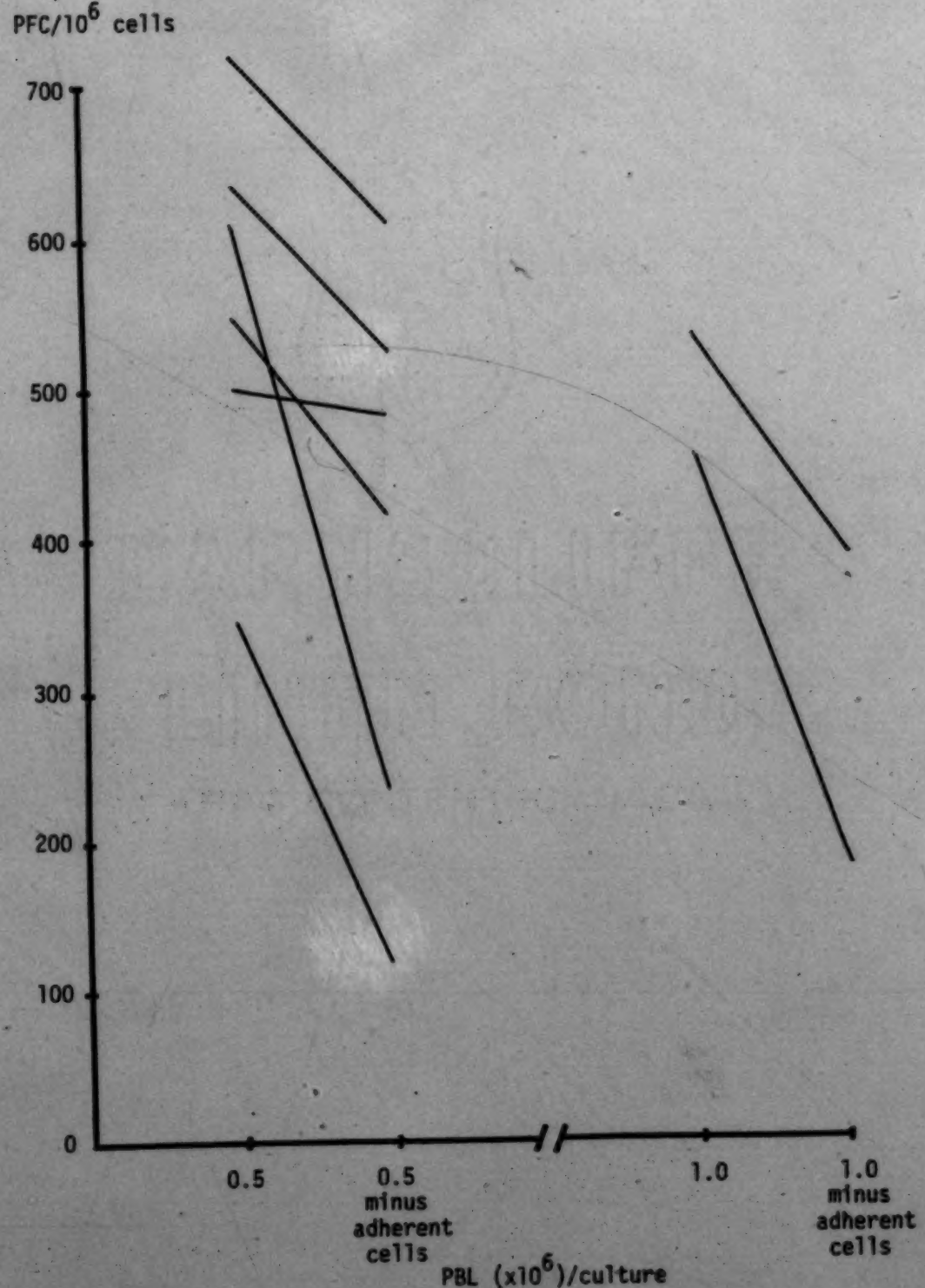
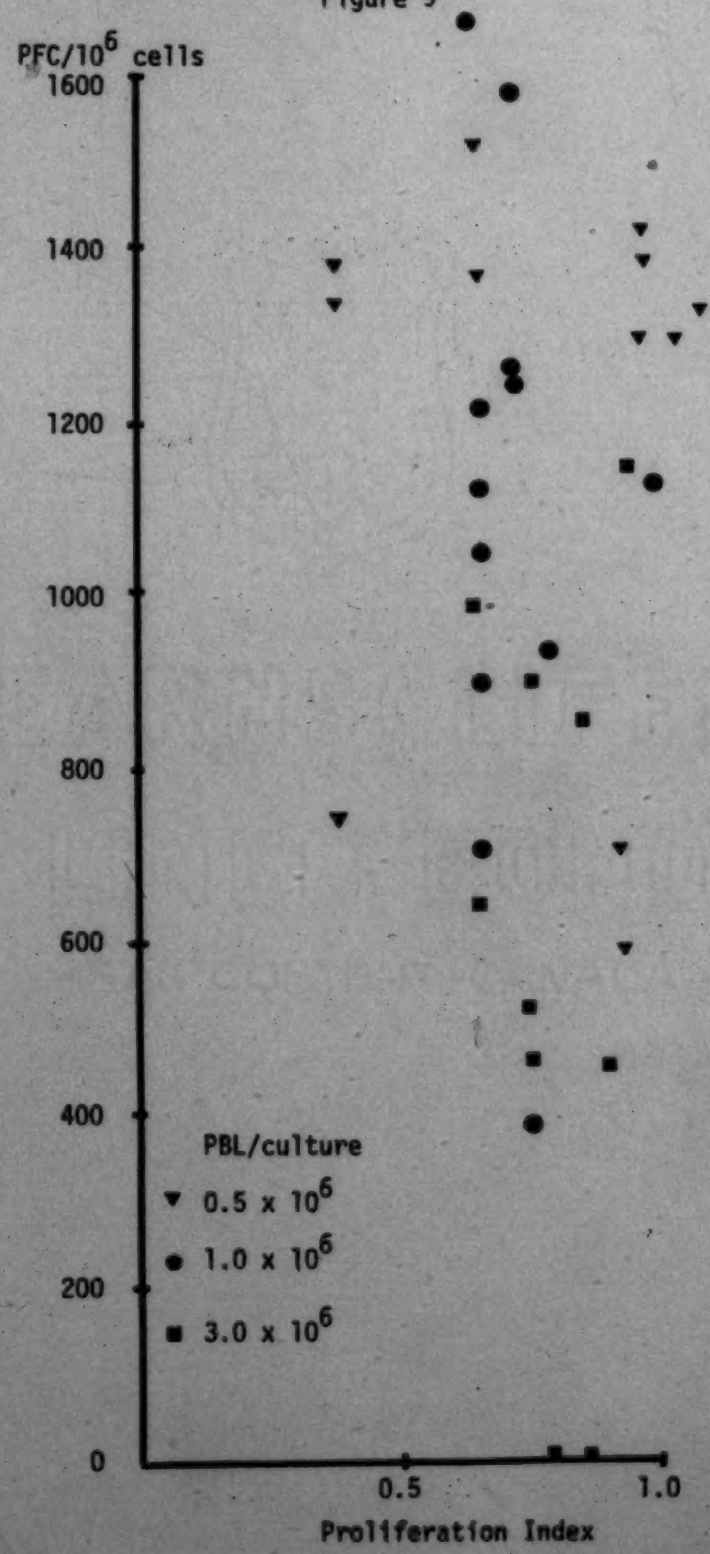

PFC/10^6 cells
700
600
500
400
300
200
100
0
0.5
0.5 minus adherent cells
1.0
1.0 minus adherent cells
PBL (x10^6)/culture

Figure 9. Effect of lymphocyte proliferation in "inducing" cultures on PFC results. Proliferation indices are plotted for cultures of reclaimed cells. All cultures were stimulated with 10^8 SRBC.

Figure 9


PFC/10^6 cells
1600
1400
1200
1000
800
600
400
200
0
PBL/culture
0.5 x 10^6
1.0 x 10^6
3.0 x 10^6
0.5
1.0
Proliferation Index

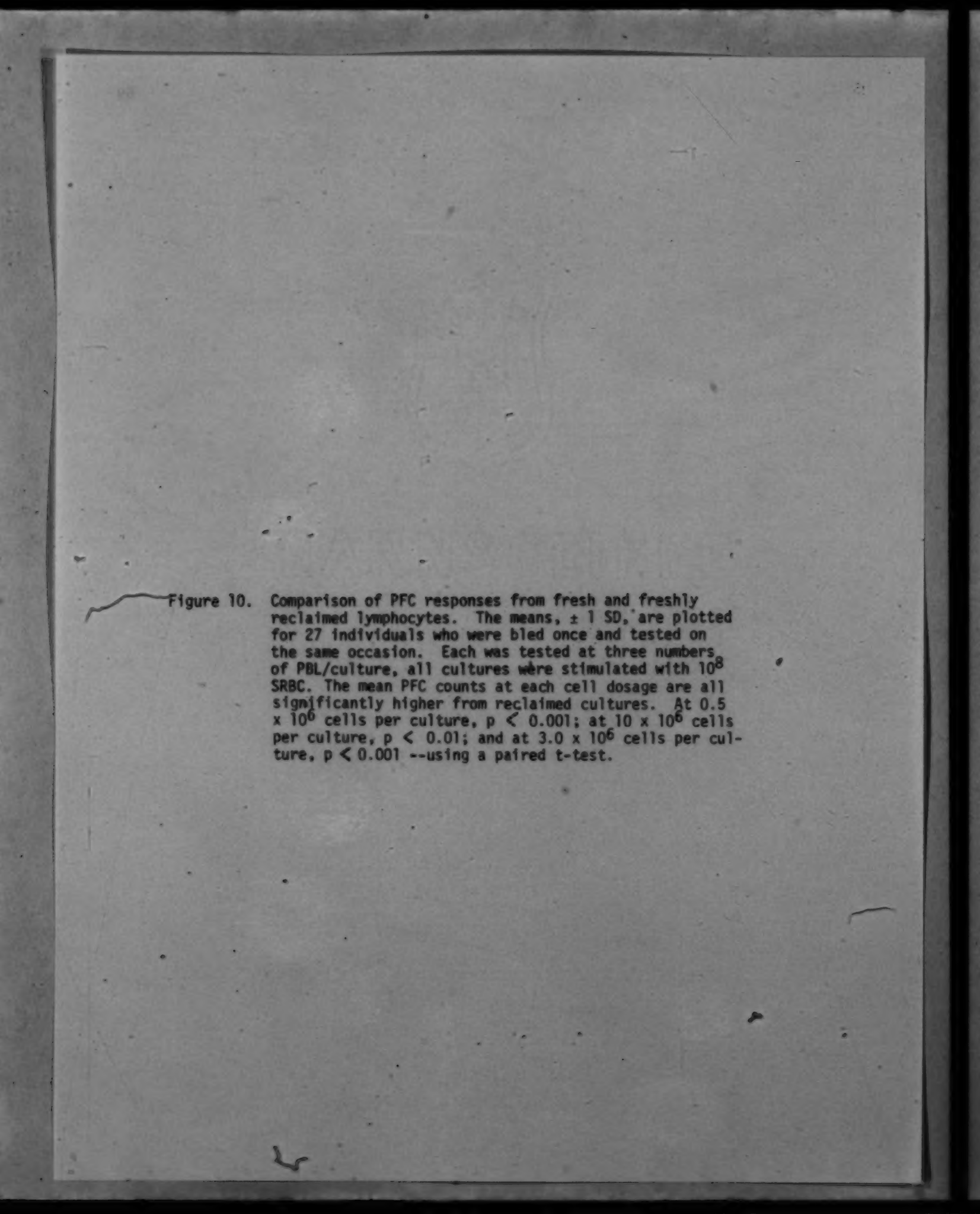


Figure 10. Comparison of PFC responses from fresh and freshly reclaimed lymphocytes. The means, ± 1 SD, are plotted for 27 individuals who were bled once and tested on the same occasion. Each was tested at three numbers of PBL/culture, all cultures were stimulated with 10^8 SRBC. The mean PFC counts at each cell dosage are all significantly higher from reclaimed cultures. At 0.5 x 10^6 cells per culture, $p < 0.001$; at 10 x 10^6 cells per culture, $p < 0.01$; and at 3.0 x 10^6 cells per culture, $p < 0.001$ --using a paired t-test.

Figure 10

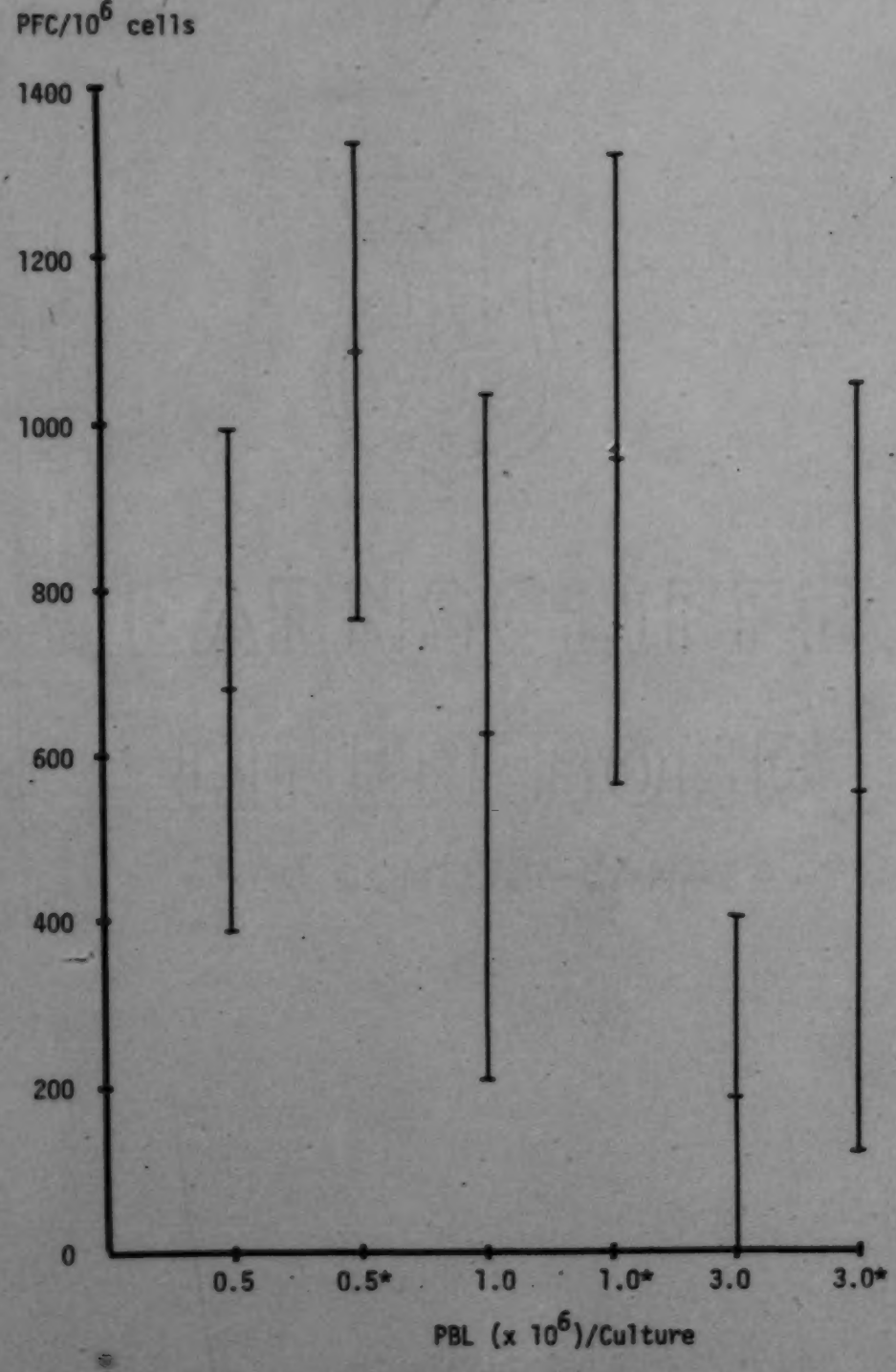

PFC/10^6 cells
1400
1200
1000
800
600
400
200
0
0.5
0.5*
1.0
1.0*
3.0
3.0*
PBL (x 10^6)/Culture
* reclaimed cells

Figure 11. Effect of adding freshly reclaimed, autologous PBL to cultures of fresh PBL, on the PFC response. Fresh and reclaimed cells from the same bleed were tested to compare the responses when cultured alone or mixed in 1:1 ratio. Five individuals were tested, the total cell numbers being 1.0×10^6 (11a), or 3.0×10^6 (11b). All cultures were stimulated with 10^8 SRBC.

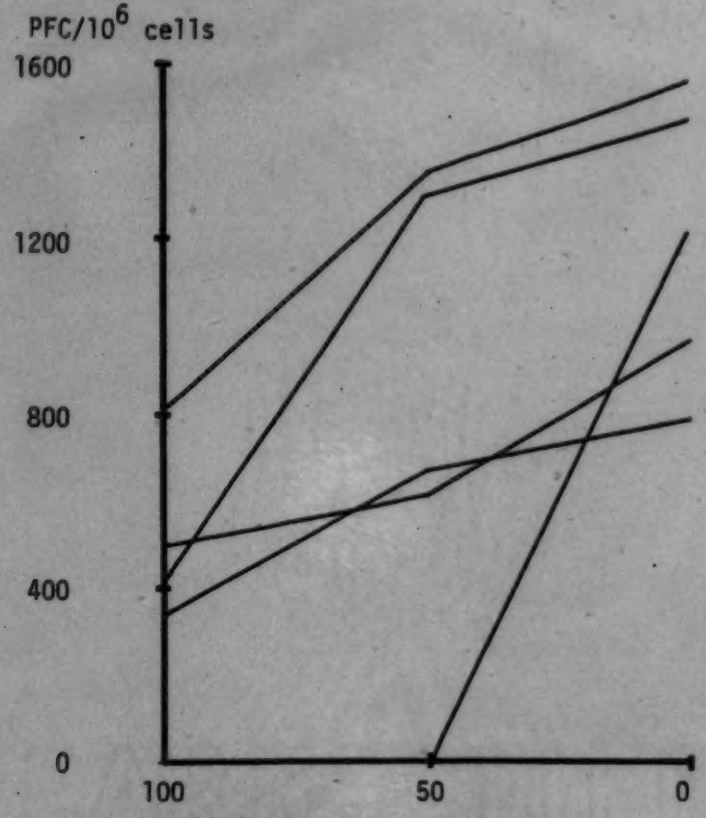


Figure 11a

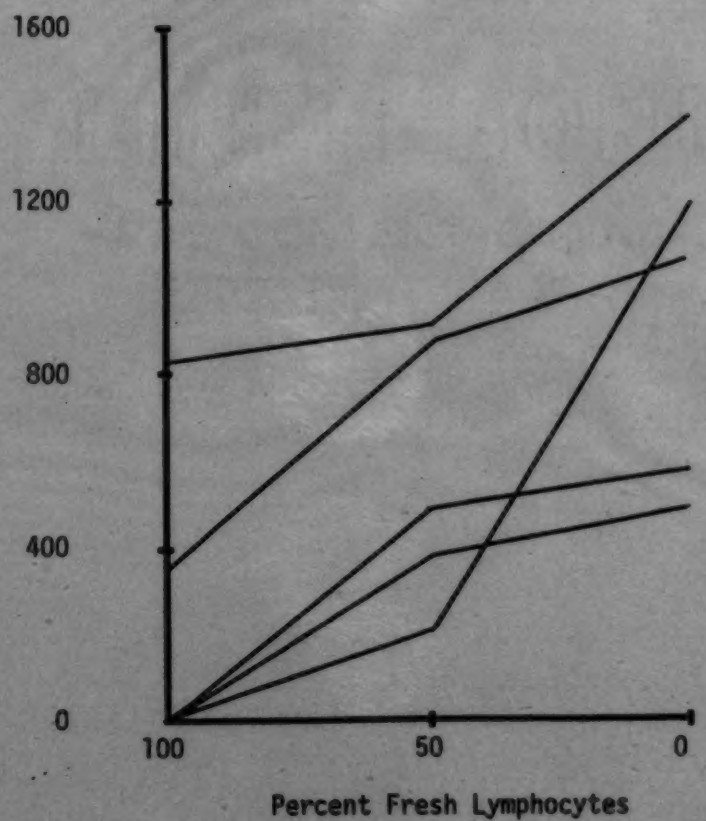


Figure 11b

Figure 12. Effect of adding freshly reclaimed, autologous PBL to cultures of fresh PBL, on the PFC response. Fresh and reclaimed cells from one bleed were mixed in varying proportions and stimulated with 10^8 SRBC. All cultures from the five individuals contained a total of 1.0×10^6 PBL.

Figure 12

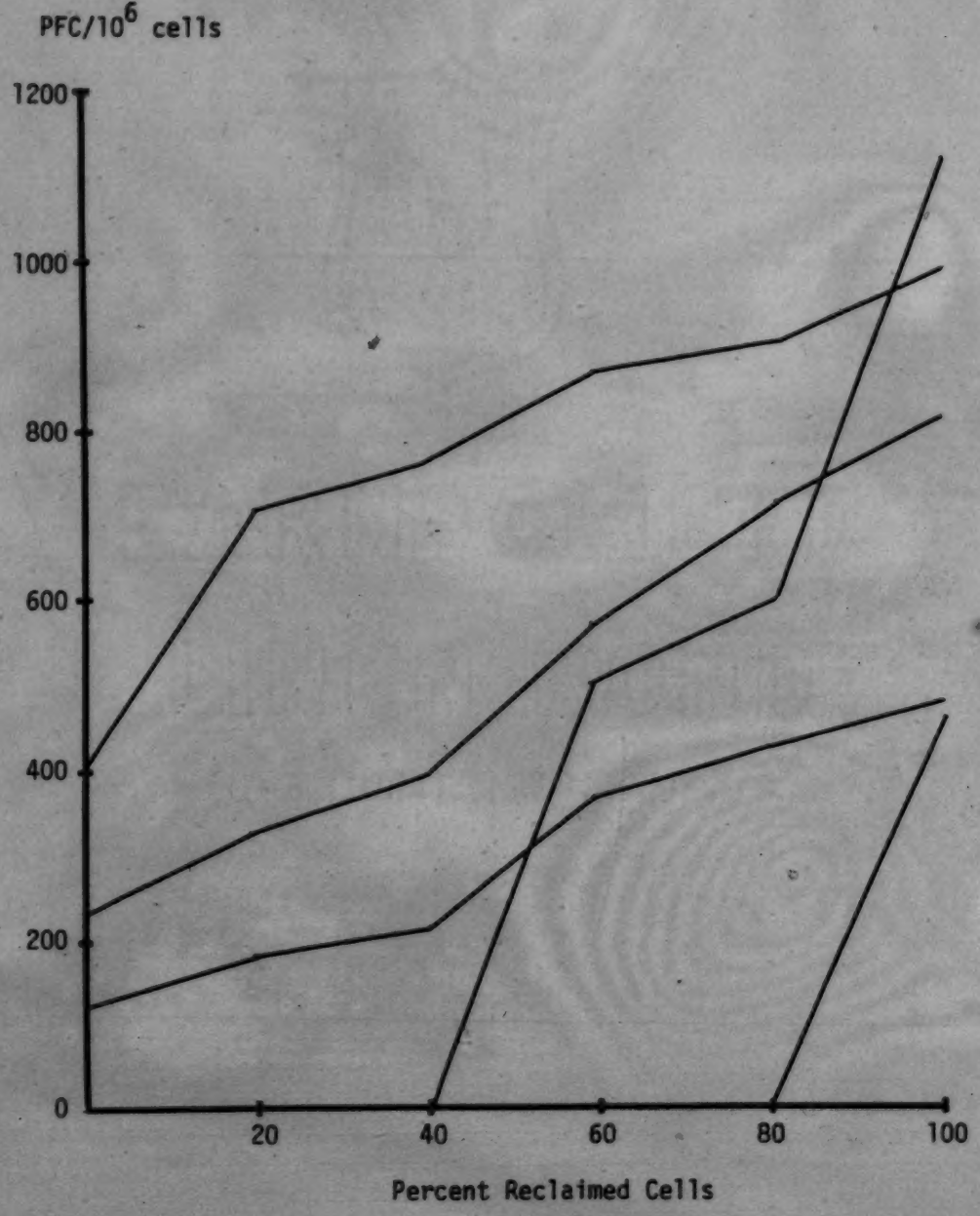

PFC/10^6 cells
1200
1000
800
600
400
200
0
20
40
60
80
100
Percent Reclaimed Cells

Figure 13. Effect of the addition of pre-incubated cells to an autologous culture on the PFC response. The means, ± 1 SD, of three experiments in which PBL were pre-incubated (primary culture) with or without antigen for three days. These cells were then added in 1:1 mixture to freshly reclaimed autologous PBL (secondary culture). The secondary cultures contained a total of 1.0×10^6 cells and were stimulated with 10^8 SRBC. The p values, comparing the control cultures (freshly reclaimed PBL) to those with added pre-incubated cells are, using a paired t-test: $p < 0.01$ for cells pre-incubated without antigen or with 10^9 SRBC, $p < 0.02$ for cells pre-incubated with 10^7 SRBC and $p < 0.4$ for cells pre-incubated with 10^8 SRBC.

Figure 13

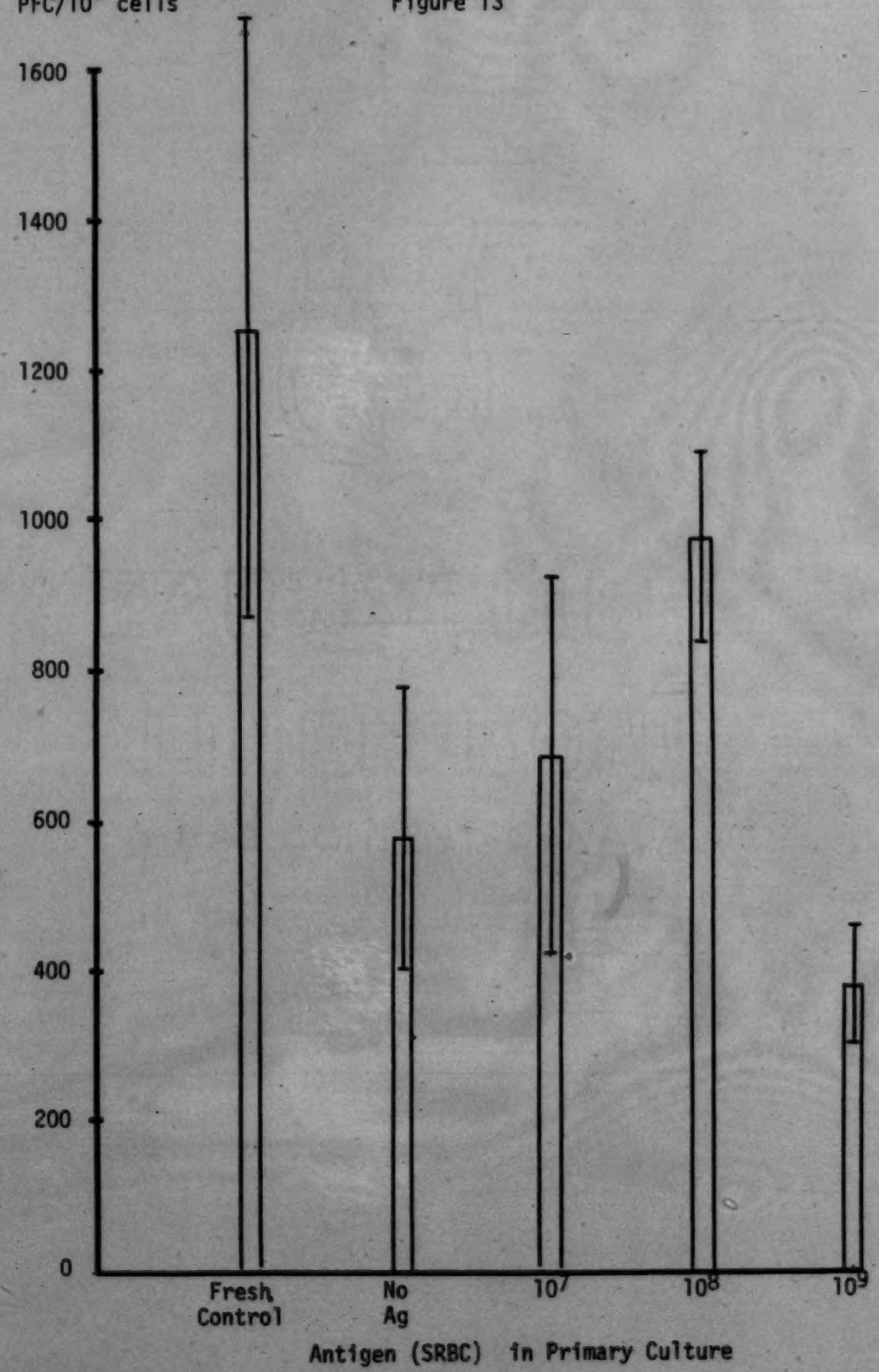

PFC/10^6 cells
1600
1400
1200
1000
800
600
400
200
0
Fresh Control
No Ag
10^7
10^8
10^9
Antigen (SRBC) in Primary Culture

Figure 14. Effect of the addition of pre-incubated cells to an autologous culture on the PFC response. The means, ± 1 SD, of six experiments in which PBL were pre-incubated (primary culture) with or without antigen for six days. These cells were then added in 1:1 mixture to freshly reclaimed autologous PBL (secondary culture). The secondary cultures contained a total of 1.0×10^6 cells and were stimulated with 10^8 SRBC.
The p values, comparing the control cultures to those with added pre-incubated cells are, using a paired t-test: $p < 0.05$ for cells pre-incubated without antigen, $p < 0.05$ for cells pre-incubated with 10^7 SRBC, $p < 0.005$ for cells pre-incubated with 10^8 SRBC, $p < 0.1$ for cells pre-incubated with 10^9 SRBC.

Figure 14

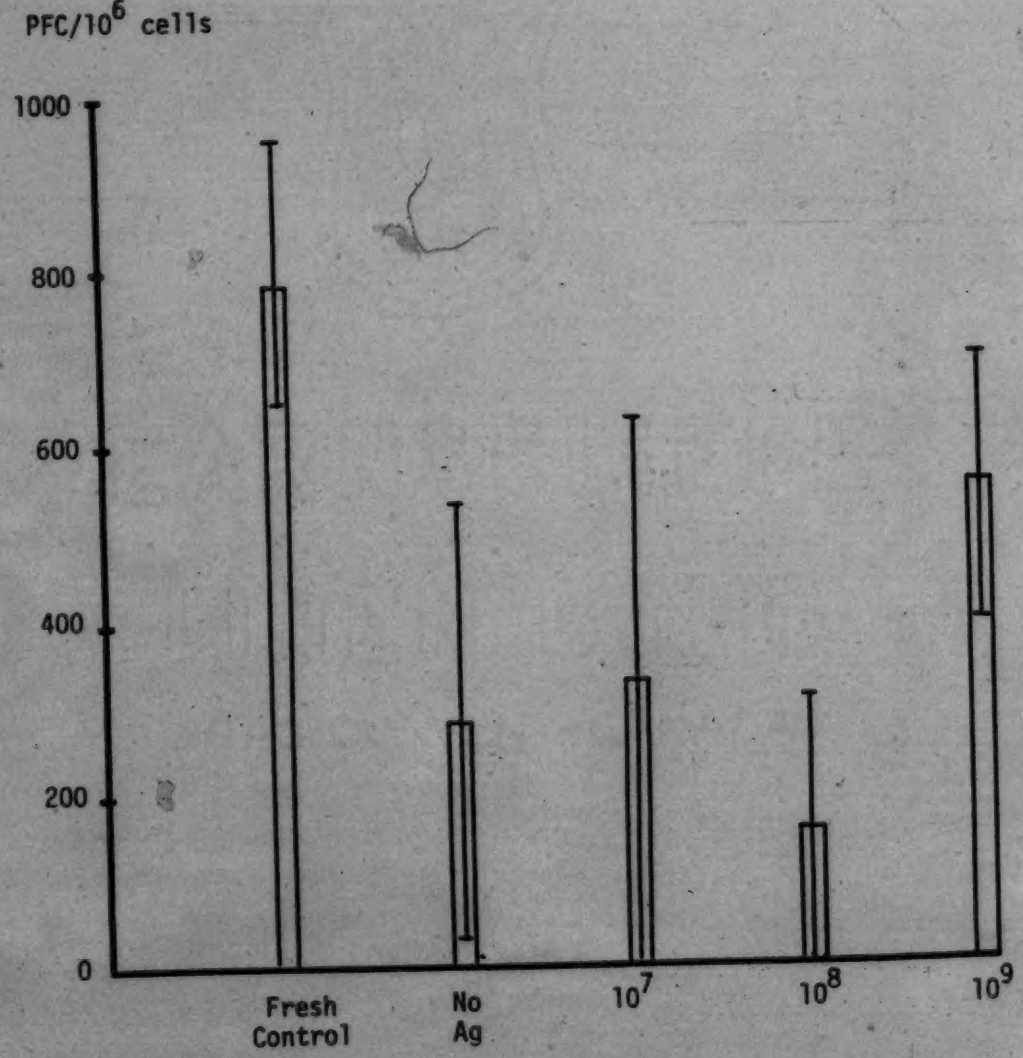

PFC/10^6 cells
1000
800
600
400
200
0
Fresh Control
No Ag
10^7
10^8
10^9
Antigen (SRBC) in Primary Culture

Figure 15. Effect of the addition of pre-incubated cells to an autologous culture on the PFC response. The means, ± 1 SD, of nine experiments in which PBL were pre-incubated for one day (primary culture) with or without antigen. These cells were then added in 1:1 mixture to freshly reclaimed autologous PBL (secondary culture). The secondary cultures contained a total of 1.0×10^6 cells and were stimulated with 10^8 SRBC. The p values, comparing the control cultures to those with added pre-incubated cells, are, using a paired t-test:

PBL		B CELLS	
Antigen in 1° Culture		Antigen in 1° Culture	
0	$p < 0.005$	0	$p < 0.005$
10^8	$p < 0.005$	10^8	$p < 0.001$
10^9	$p < 0.005$	10^9	$p < 0.001$

Figure 15

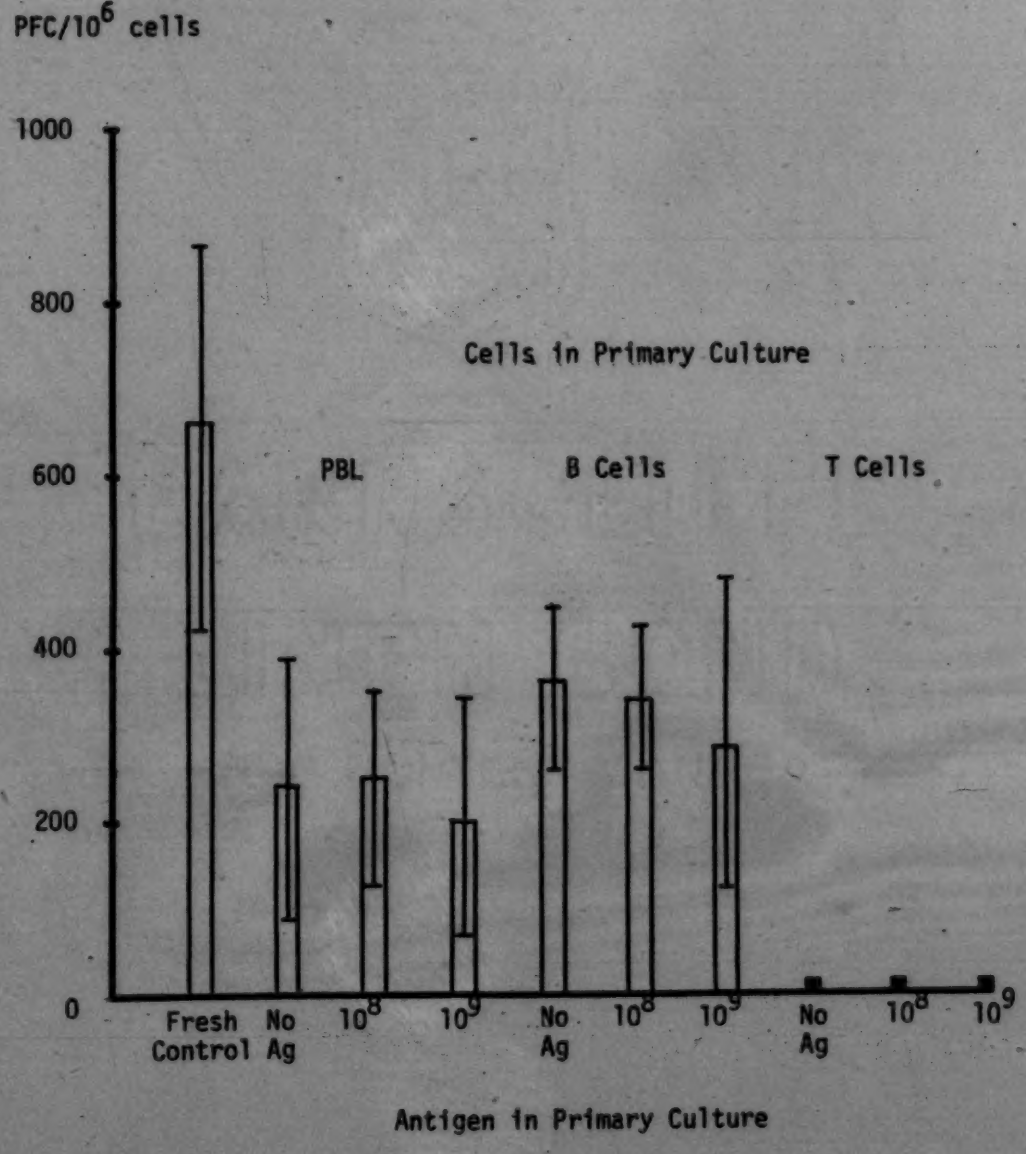
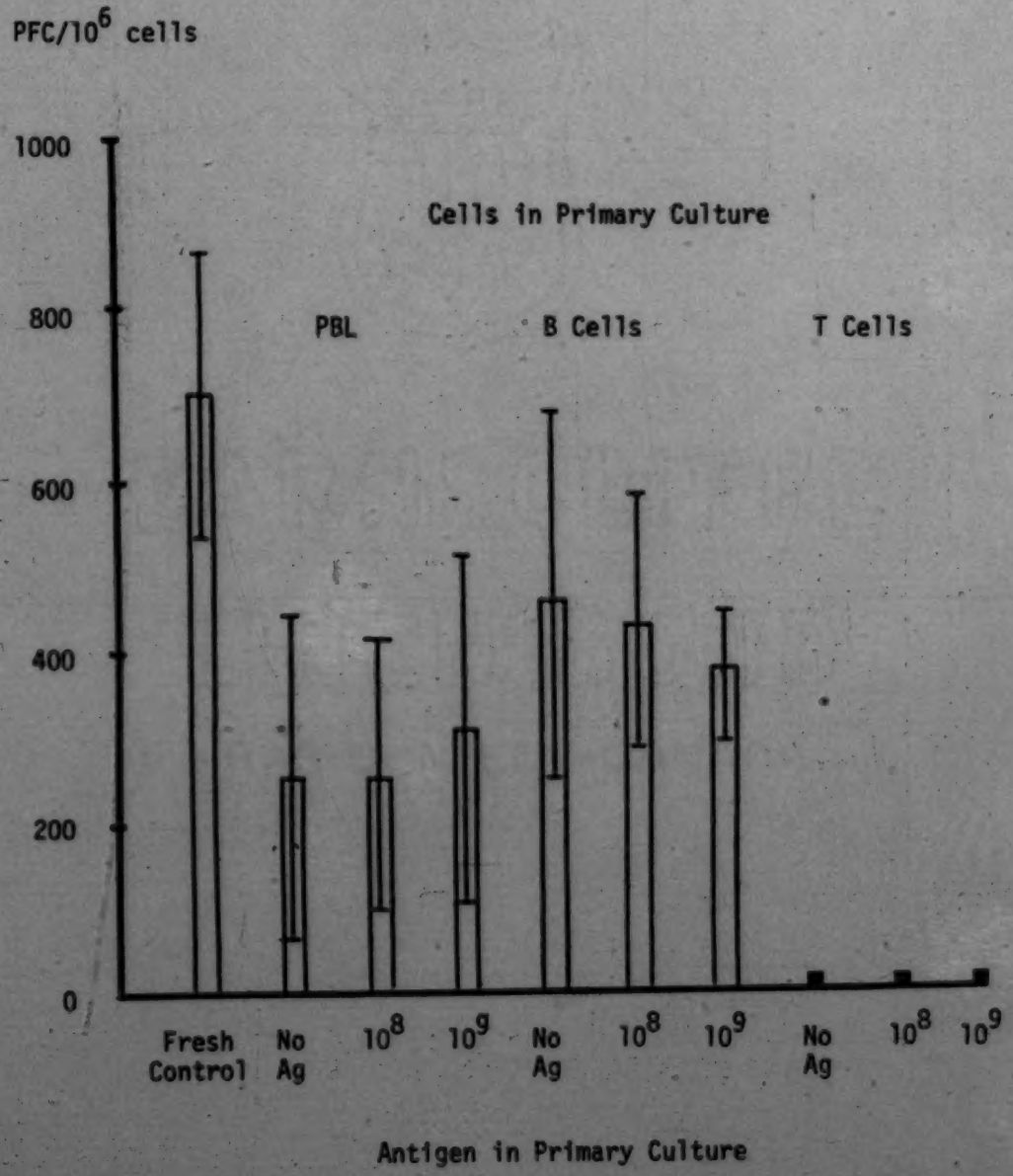

PFC/10^6 cells
1000
800
600
400
200
0
Cells in Primary Culture
PBL
B Cells
T Cells
Fresh Control
No Ag
10^8
10^9
No Ag
10^8
10^9
No Ag
10^8
10^9
Antigen in Primary Culture

Figure 16. Effect of the addition of pre-incubated cells to an autologous culture on the PFC response. The means, ± 1 SD, of six experiments in which PBL were pre-incubated for six days (primary culture) with or without antigen. These cells were then added in 1:1 mixture to freshly reclaimed autologous PBL (secondary culture). The secondary cultures contained a total of 1.0×10^6 cells and were stimulated with 10^8 SRBC.
The p values, comparing the control cultures to those with added pre-incubated cells, are; using a paired t-test:

PBL		B CELLS	
Antigen in 1° Culture		Antigen in 1° Culture	
0	$p < .005$	0	$p < 0.025$
10^8	$p < .005$	10^8	$p < 0.01$
10^9	$p < 0.01$	10^9	$p < 0.01$

Figure 16


PFC/10^6 cells
1000
800
600
400
200
0
Cells in Primary Culture
PBL
B Cells
T Cells
Fresh Control
No Ag
10^8
10^9
No Ag
10^8
10^9
No Ag
10^8
10^9
Antigen in Primary Culture

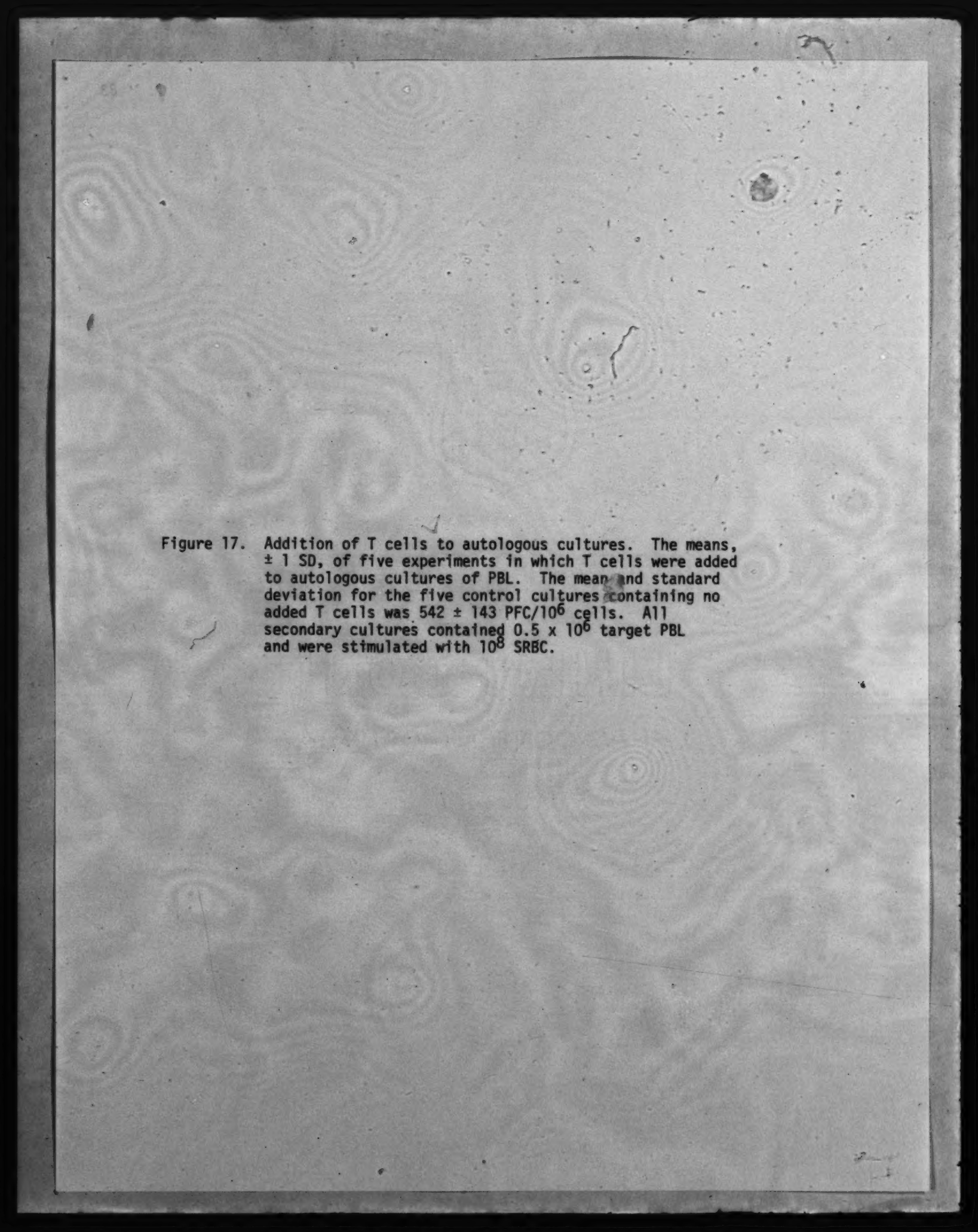


Figure 17. Addition of T cells to autologous cultures. The means, ± 1 SD, of five experiments in which T cells were added to autologous cultures of PBL. The mean and standard deviation for the five control cultures containing no added T cells was 542 ± 143 PFC/10^6 cells. All secondary cultures contained 0.5 x 10^6 target PBL and were stimulated with 10^8 SRBC.

Figure 17

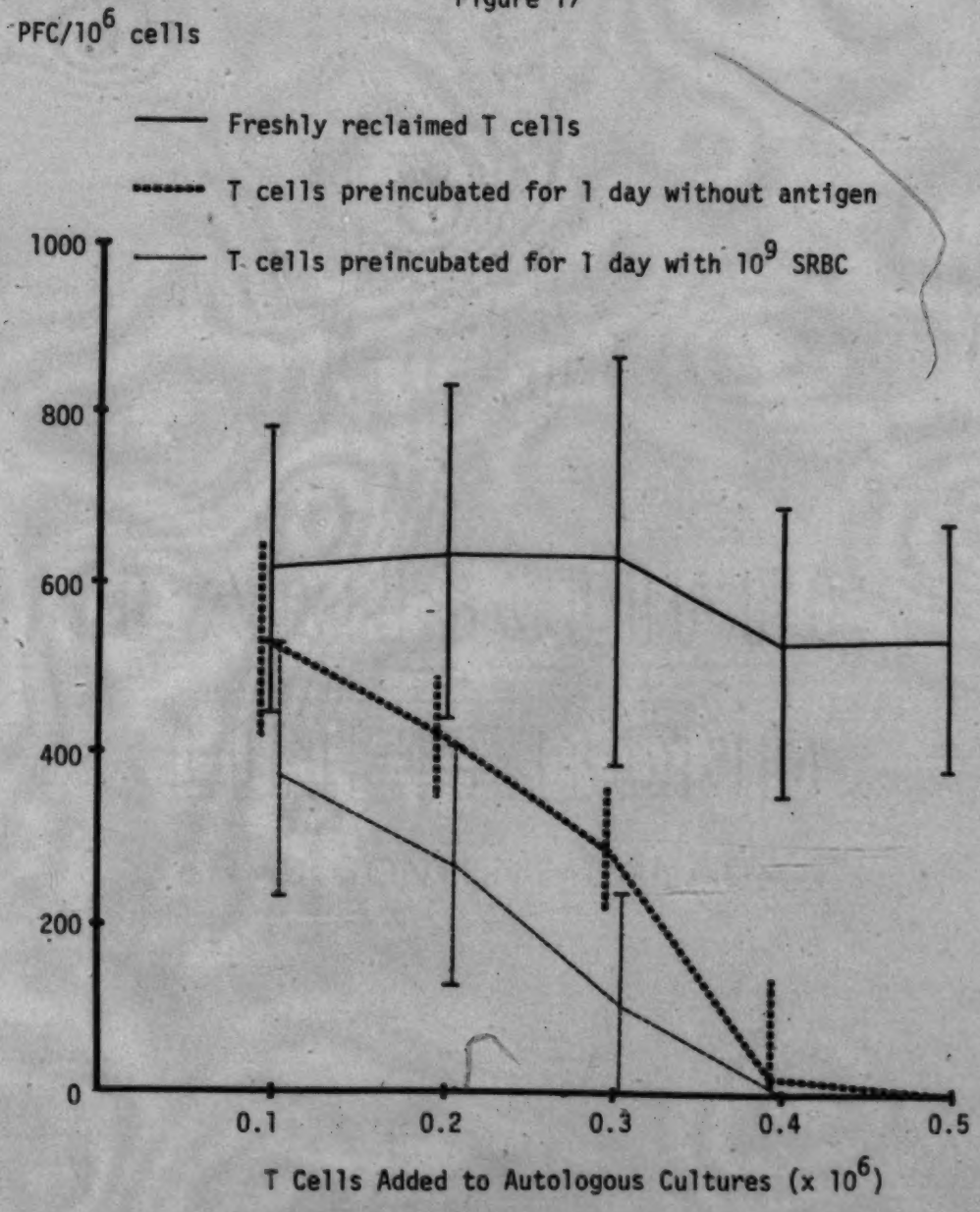
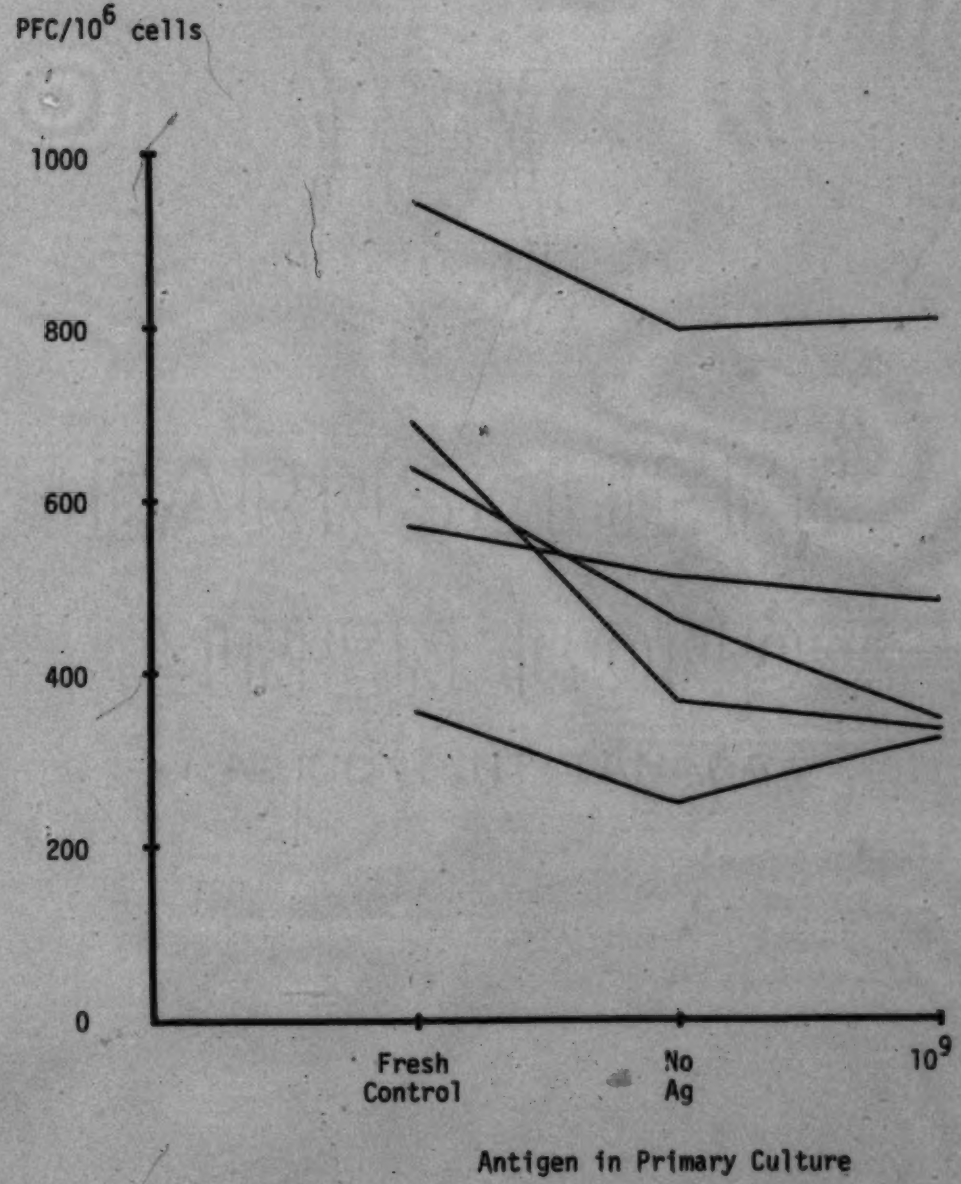

PFC/10^6 cells
Freshly reclaimed T cells
T cells preincubated for 1 day without antigen
T cells preincubated for 1 day with 10^9 SRBC
1000
800
600
400
200
0
0.1
0.2
0.3
0.4
0.5
T Cells Added to Autologous Cultures (x 10^6)

Figure 18. Effect of the addition of pre-incubated adherent cells to an autologous culture on the PFC response. Five experiments in which adherent cells were pre-incubated with or without antigen for 1 day. These cells were then added in 1:1 mixture to freshly reclaimed autologous PBL (secondary culture). The secondary cultures contained a total of 1.0×10^6 cells and were stimulated with 10^8 SRBC. The means, $\pm$ ISD are: control cultures, 565 ± 46 PFC/10^6 cells; cultures with adherent cells pre-incubated without antigen, 409 ± 119 PFC/10^6 cells; and cultures with adherent cells pre-incubated with 10^9 SRBC, 383 ± 91 PFC/10^6 cells. The p values, comparing control cultures to those with added pre-incubated adherent cells, are: $p < 0.05$ for cells pre-incubated without antigen and $p < 0.01$ for cells pre-incubated with 10^9 SRBC; using a paired t-test.

Figure 18


PFC/10^6 cells
1000
800
600
400
200
0
Fresh Control
No Ag
10^9
Antigen in Primary Culture

TABLES

Table 2

Purity of Cell Preparations

(a) SRBC - Rosetting

T Cells		B Cells	
% Rerosetting	% Kill (Anti-B)	% Rerosetting	% Kill (Anti-B)
91 ± 7.3	4.1 ± 1.3	5.2 ± 1.1	60 ± 3.5
n=9	n=9	n=5	n=5

(b) Nylon Wool Column

T Cells		B Cells	
% Rosetting	% Kill (Anti-B)	% Rosetting	% Kill (Anti-B)
89 ± 10.3	5.0 ± 3.3	7.3 ± 22	63 ± 2.3
n=5	n=9	n=5	n=5

Table 3

Effect of Lymphocyte Treatment with 25 µg Mitomycin C on the PFC Response

PFC/10^6 Cells			
0.5 x 10^6 Cells/Culture		1.0 x 10^6 Cells/Culture	
Untreated	Treated *	Untreated	Treated
306 ± 31	193 ± 31	213 ± 23	126 ± 23
513 ± 46	406 ± 30	460 ± 53	300 ± 40
540 ± 60	406 ± 50	366 ± 23	300 ± 40
366 ± 23	313 ± 33	313 ± 33	286 ± 23

*The p values, comparing treated and untreated cultures, are all < 0.01, using a paired t-test.

Table 4

Variation Between Experiments

Means and 1 SD for 4 days on which 4 individuals' PBL were tested at 1.0×10^6 PBL/culture, and 10^8 SRBC/Culture.

	PFC/10^6 Cells
Day 1	729 ± 224
Day 2	858 ± 124
Day 3	406 ± 198
Day 4	583 ± 83

Comparing Day 2 and Day 3, $p = 0.05$. Differences between means of experiments on other days are not significant. ($p < 0.4$)

Table 5

Variation Within an Experiment

Means and 1 SD of five replicate cultures from each of six individuals. Each mean is therefore the average of 15 wells.

PFC/10^6 Cells

	0.5×10^6 PBL/Culture 10^8 SRBC	1.0×10^6 PBL/Culture 10^8 SRBC
1	763 ± 116	374 ± 75
2	1706 ± 89	0
3	1392 ± 64	932 ± 97
4	645 ± 26	1179 ± 101
5	427 ± 30	785 ± 80
6	1019 ± 100	493 ± 54

Table 6

Day to Day Variation in 20 Individuals,
Each Tested on 4 Occasions

Each culture contained 1.0×10^6 cells
and was stimulated with 10^8 SRBC

PFC/10^6 Cells

		SD		SD		SD		SD	Mean ± 1 SD	p for high and low values
1	373	33	540	40	713	17	286	11	478 ± 189	<0.005
2	466	31	500	43	353	23	880	43	549 ± 229	<0.005
3	846	15	540	15	393	25	546	44	581 ± 190	<0.01
4	840	20	973	23	353	27	426	11	648 ± 285	<0.001
5	540	11	620	21	793	19	740	23	673 ± 114	<0.025
6	626	40	386	11	1233	15	413	31	664 ± 398	<0.001
7	166	17	620	22	653	22	1006	22	611 ± 344	<0.001
8	646	33	900	31	880	31	826	33	813 ± 74	<0.05
9	1033	37	740	33	633	27	973	30	844 ± 189	<0.025
10	633	15	760	31	600	33	ND		664 ± 84	<0.05
11	940	15	533	37	1033	29	ND		835 ± 265	<0.01
12	1153	21	373	15	533	13	ND		686 ± 411	<0.001
13	826	23	336	19	506	11	0		417 ± 250	
14	1033	11	453	23	646	11	0		533 ± 429	
15	273	31	606	24	0		133	11	253 ± 318	
16	826	31	1013	23	1360	44	0		799 ± 577	
17	386	14	373	31	285	30	306	19	337 ± 49	<0.05
18	833	20	620	30	0		393	23	461 ± 352	
19	440	23	413	27	579	19	600	25	508 ± 95	<0.05
20	346	20	1033	11	0		540	20	493 ± 289	

all 80 experiments 564 ± 206

Table 7

Comparison of T/B Cell Separations by
SRBC Rosetting and by Nylon Wool Column Adherence

(PFC/10^6 Cells)

All cultures contained 1.0×10^6 cells; 10^8 SRBC.

% T Cells	E-RFC Separation				Nylon Wool Column Separation			
	*#1	SD	#2	SD	#1	SD	#2	SD
0	0	0	0	0	0	0	0	0
10	346	31	253	22	400	30	243	37
20	460	23	0	0	423	33	120	33
50	413	34	1233	27	397	23	1019	30
80	446	27	813	33	495	20	813	40
90	1266	44	0	0	1166	27	0	0
100	0	0	0	0	0	0	0	0
Not Separated	753	30	1153	37				

The percentage of T cells represents the proportion of T cells added to B cells to total 1.0×10^6 lymphocytes in each culture.

*#1 and #2 are different individuals.

p values comparing E-RFC Separation and Nylon Wool Column Separation. (paired t-test)

% T CELLS	#1	#2
10	< 0.4	< 0.4
20	< 0.2	---
50	< 0.1	< 0.2
80	< 0.4	---
90	< 0.2	---

Table 8

PFC/10^6 Cells and Proliferation Indices VS % T Cells

All cultures contained 1.0 x 10^6 cells; 10^8 SRBC

% T Cells	PFC[1]	PFC[2]	PFC[3]	PFC[4]	PFC[5]	PFC[6]	PFC[7]	PFC[8]	PFC[9]	PFC[10]	PFC[11]	Mean ± Standard Deviation
0	0	0	0	0	0	0	0	0	0	0	0	0
10	0	0	606	0	0	0	486	0	346	253	0	146±223
20	0	306	393	533	833	0	906	0	460	0	946	397±375
50	233	373	626	520	613	493	980	766	413	1233	666	628±284
80	413	few	846	626	573	546	446	446	446	813	0	477±257
90	0	few	566	593	386	0	566	0	1266	0	233	346±387
100	0	0	0	0	0	0	0	0	0	0	0	0
Not Separated	0	793	880	633	740	800	906	0	753	1153	1033	

The percentage of T cells represents the proportion of T cells added to B cells to total 1.0 x 10^6 lymphocytes in each culture. Cell separations were performed by SRBC-rosetting.

#1 - #11 represent eleven different individuals

Table 9

Variation Between Experiments - Reclaimed Cells From Different Bleeds

Cells from four individuals were frozen and reclaimed for testing 1 - 3 weeks later. Cultures were stimulated with 10^8 SRBC.

PBL ($x10^6$) per culture	Experiment 1	2	3	Mean ± 1 SD	Fresh Responses Mean ± 1 SD	p values for the values making up "reclaimed" and "fresh" cell means
	PFC/10^6 cells					
.5	926	786	746	819 ± 94	646 ± 233	< 0.01
1.0	620	446	533	533 ± 87	320 ± 180	< 0.01
3.0	200	346	366	304 ± 90	0	----
.5	846	813	746	801 ± 50	720 ± 196	< 0.05
1.0	406	493	386	428 ± 56	386 ± 176	< 0.05
3.0	333	366	370	356 ± 20	0	----
.5	800	773	673	748 ± 66	620 ± 193	< 0.025
1.0	633	660	593	628 ± 33	400 ± 120	< 0.01
3.0	446	533	433	470 ± 54	286 ± 176	< 0.005
.5	540	646	533	573 ± 63	320 ± 146	< 0.025
1.0	473	500	366	446 ± 70	200 ± 160	< 0.005
3.0	413	540	400	451 ± 22	0	----

Table 10

Effect of DMSO Treatment and Freezing on PFC Responses by PBL

PBL/culture ($x10^6$)	Treatment	PFC/10^6 Cells Individual 1	SD	2	SD
	cultured as usual				
0.5		460	23	126	15
1.0		400	20	226	11
3.0		0		0	
0.5	20% DMSO; added for less than 5 mins.; cells washed, cultured	580	22	333	22
1.0		500	31	413	20
3.0		333	37	0	
0.5	20% DMSO; cells frozen, reclaimed, cultured	566	40	353	15
1.0		493	20	493	11
3.0		346	15	0	

the p values

Individual	1	2	PBL/Culture ($x 10^6$)
Control vs	< 0.05	< 0.005	0.5
DMSO Treated	< 0.1	< 0.01	1.0
	---	---	3.0
Control vs	< 0.1	< 0.005	0.5
Frozen, reclaimed	< 0.1	< 0.005	1.0
	---	---	3.0

Table 11

Suppressor Indices* in Cultures with Added Pre-Incubated **T Cells

Responding cultures contain 0.5×10^6 target cells; 10^8 SRBC

Antigen in 1^0 Culture	T Cells Added ($\times 10^6$)	Individuals 1	2	3	4	5
None	.1	8	25	24	20	2
None	.2	29	34	50	30	15
None	.3	82	60	56	46	36
None	.4	100	100	100	100	65
None	.5	100	100	100	100	100
10^9	.1	12	55	32	28	60
10^9	.2	29	74	50	45	67
10^9	.3	54	100	100	53	100
10^9	.4	100	100	100	100	100
10^9	.5	100	100	100	100	100
None	Number of T Cells added to reach a 50% Suppression Level	.25	.28	.20	.31	.38
10^9		.29	<.1	.20	.29	<.1

* Suppressor Index = $\left[1 - \frac{\text{Added T cells, pre-incubated}}{\text{Added T cells, fresh}}\right] 100$

** All preincubations were 24 hours

Paired t-test values; 5 experiments combined. The p values comparing presence with absence of SRBC in the primary culture.

T cells Added ($\times 10^6$)	
0.1	< 0.05
0.2	< 0.05
0.3	< 0.05
0.4	Not Significant
0.5	Not Applicable

Table 12

Addition of T Cells Pre-incubated Without Antigen in Various Media to Cultures of Autologous PBL

(PFC/10^6 Cells)

All secondary cultures contained 1.0×10^6 cells; 10^8 SRBC

Control (No pre-incubated cells)		RPMI-1640 + AB serum	McCoy's alone		RPMI + Autologous Serum	10^8 SRBC Added after 1 Day*
	SD			SD		
533	30	0	440	33	0	0
626	20	0	533	24	0	0
696	31	0	500	20	0	0
553	15	0	393**	37	0	0

* In this experiment the pre-incubated cells were PBL. The primary culture contained RPMI-1640 and AB serum.

**Control experiment in which RPMI-1640 was substituted for McCoy's medium. In this experiment 4 SLE sera were also used in primary cultures. No PFC responses were found in these cultures.

IV. DISCUSSION AND CONCLUSIONS

A. Discussion

Several goals prompted our efforts to standardize a technique for the *in vitro* sensitization of human PBL.

In the mouse, genetic control of the primary immune response to synthetic polypeptide and protein antigens has been shown to be associated with the MHC and to exist at the IgM level. (McDevitt et al, 1972; Merryman et al, 1977).

Genetic analysis in human studies has been difficult because of the lack of a reproducible and standardized technique to measure immune responses *in vitro*.

A standardized technique to study normal antibody production might be used to study the aberrant immune responses in pathological conditions (Dosch et al, 1978; Waldmann and Broder, 1977).

A technique for *in vitro* sensitization of lymphocytes would also be useful for studies of cellular ontogeny, cell cooperation, and antigen recognition and processing.

This study began with a survey of 27 individuals so that the technique could be standardized with respect to antigen and lymphocyte dosages, and the kinetics of the response.

As described in the results, cultures containing different numbers of lymphocytes were incubated in the presence of antigen, and harvested after different time intervals - from day one to day fourteen. The results represented in Figure 1 indicate that the optimal length of sensitization was six days. No responses were observed before day 3 or after day 8. Low numbers of PFC developed at days 3, 4 and 8 and moderate numbers at days 5 and 7.

To study the effect of varying amounts of antigen per culture on the PFC response, 10^7, 10^8, or 10^9 SRBC were added to replicate cultures of lymphocytes. The optimum amount of antigen per culture was 10^8 SRBC (Figures 2 and 3). In this series of experiments moderate PFC responses were observed at 10^7 and 10^9 SRBC per culture, although in our first series (Figure 1), these dosages of antigen did not cause any response. This may have been because different individuals were used in the two studies, not all persons may respond over the full range of antigen dosages used.

The effect of the number of lymphocytes in culture on the PFC results is compared in Figure 4. 0.5×10^6 or 1.0×10^6 PBL/culture was the optimal lymphocyte dose. Thus, the 100:1 SRBC:PBL ratio gives optimal responses in our system.

Dosch and Gelfand (1977) reported the optimal ratio to be between 10:1 and 1:1. Uytde Haag and Heijnen (1978) also report an optimal ratio of 1:1 using this technique. In all these experiments the peak PFC response was observed on day 6.

Using TNP-PAA to stimulate PBL in Mishell-Dutton cultures, the peak response was observed on day 8 (Delfraissy et al, 1977). So, when comparing <u>in vitro</u> generated PFC results, one must take into consideration the antigen and the culture system used.

When volume and cell concentrations of cultures were varied, there was evidence that cell density is a factor in obtaining optimal responses from an individual. Figure 5 shows that as the number of cells increased, the number of PFC increased only if there was a concomitant increase in culture volume. The decrease in PFC observed with increasing cell numbers may be due to cell crowding and lack of

nutritional supplements in those cultures where cell density was too high.

To examine the requirement for DNA synthesis during PFC production, some lymphocytes were treated with mitomycin C prior to culture. Experiments where PBL were treated with 25 ug Mitomycin C show that although some cell division must be required for development of the full PFC response, some cells will produce antibody to SRBC in vitro without DNA synthesis (Table 3). The cellular requirement for these plaques was shown; whenever new batches of AB serum or complement were used, they were tested without lymphocytes on the monolayer to ensure the absence of pseudo plaques. Although not repeated on every test plate, this may be considered sufficient evidence for their absence.

Studies of variation between experiments showed that although there is some difference in mean responses on different days, there were never days when all the responders gave high PFC counts or when all the responses were low (Table 4).

When 20 individuals were tested on four occasions each, the variation between individuals was less than that observed for different experiments for an individual (Table 6). Thus, our interest was focused on the great day to day variability that was observed with each individual.

Although previous experiments (Table 3) indicated that a certain number of PFC can occur even in the absence of DNA synthesis, we wondered whether day to day variation in the responses of one individual could, at least partly, be accounted for by varying amounts of cell proliferation occuring in culture. Proliferation indices were calculated as described in materials and methods and compared with PFC/10^6 cells (Figure 6 and 7). As can be seen from these figures, no significant correlation was found between the amount of proliferation and PFC responses for the majority of the experimental groups. Only at one lymphocyte dosage (0.5×10^6 PBL/culture) was moderate

correlation between these two parameters found ($r = 0.69$).

The cellular requirements for the generation of an optimal PFC response were investigated.

Removal of adherent cells, although not eliminating the response, showed that monocytes are required for the optimal response (Figure 8), in agreement with Dosch and Gelfand (1977) who found an enhancing effect with addition of 5 - 10% adherent cells, but did not study adherent cell depleted cultures.

Delfraissy et al (1978), using TNP-PAA to stimulate in Mishell-Dutton cultures, found that macrophages are required very early in the response. Only minimal PFC results were obtained when 1 mg silica was added to cultures on day 0. When the silica was added on day 2, the responses were significantly higher than in those cultures which received silica on day 0. They also observed that adherent cell depletion did not modify the response in a consistent fashion when plastic petri dishes were used for the cell separation (Delfraissy et al, 1977).

Similar results were obtained with tonsillar lymphocytes (Watanabe, 1974). These experiments found a requirement for macrophages in the generation of an anti-hapten response.

These different results with adherent cell depletion may represent technical differences and the important conclusion is that monocytes, at least in small numbers, are required for the generation of an optimal PFC response in vitro.

It has been demonstrated previously that although plaque formation is the function of B cells, the participation of helper T cells is necessary (Fauci et al, 1976).

We performed several experiments using separated T and B cells that were placed in the cultures in different proportions. Table 8 shows that neither T nor B cells alone will form PFC. Cultures containing 50% and 80% T cells consistently gave high responses, some individuals gave very low or no responses outside this range. Again, the proliferation indices did not correlate with high and low responses.

To exclude the possibility that the method of cell separation, namely, SRBC - rosetting, might have an effect on our results, (since SRBC are test antigen in our system) another method of T and B cell separation (nylon wool, see materials and methods) was used and the results were compared in two experiments (Table 7). Very little difference was observed, responses being slightly higher in one technique, others slightly lower.

Polyclonal induction of PFC also requires the presence of T and B cells. (Friedman et al, 1976).

To test for reproducibility, we decided to compare responses of lymphocytes of the same individual from different bleeds and cryopreserved. These cells were then reclaimed and cultured with 10^8 SRBC in three experiments. Table 9 shows that frozen cells gave much more reproducible results than the fresh cells. In these experiments the standard deviations from three occasions were all less than 95. Again, the responses from frozen, reclaimed lymphocytes were not correlated with proliferation indices (Figure 9).

The responses from 27 individuals were compared using fresh and reclaimed cells from the same bleed (Figure 10). The mean responses from cultures of 0.5×10^6, 1.0×10^6, and 3.0×10^6 PBL stimulated with 10^8 SRBC were all significantly higher in the reclaimed group ($P < .001$)

To investigate the possibility that freezing selectively eliminates a particular cell population, possibly suppressor cell, fresh lymphocytes and reclaimed PBL were added together in culture. The responses of mixed, 1:1 ratio, cultures were always intermediate between those of the fresh cell cultures, (low) and those of the reclaimed cell cultures (high) (Figure 11).

More extensive experiments with varying ratios of fresh and reclaimed cells showed linearly increasing responses from cultures of 100% fresh lymphocytes, to cultures of 100% reclaimed lymphocytes (Figure 12).

Two individuals who gave no responses with fresh lynphocyte cultures gave significant responses when lymphocytes from the same bleed were frozen, reclaimed, and then cultured. One of these individuals gave the highest response of the experiment with 100% reclaimed lymphocytes. This suggests the possibility of the action of suppressor cells in the fresh cell population, and for their removal by freezing.

Since the freezing procedure involves the DMSO treatment of lymphocytes, the effect of DMSO treatment alone, without freezing, was studied (Table 10). These results suggest that further experiments would be worth performing to see if this tendency toward increased response by DMSO alone would become significant biologically. These results cannot be considered biologically significant since there are not sufficient data to say that the results from cultures without DMSO have less than 5% probability of coming from a common population made up of results from the two DMSO treated groups.

These experiments indicated the involvement of suppressor cells in PFC development in vitro. Variation in the numbers of suppressor cells present in the circulation of each individual at any particular time could, at least partly, be responsible for the observed day to day variability of PFC responses by the same individual, as was discussed earlier.

These observations prompted a further series of experiments where we attempted to induce suppressor cell activation <u>in vitro</u>.

Lipsky et al, 1978, and Shore et al, 1978, have demonstrated that pre-incubation with antigen results in development of significant suppressor cell activity in systems measuring pokeweed mitogen responsiveness and anti-ovalbumin PFC responses in humans.

In our first series of experiments, PBL were pre-incubated for three days with 0, 10^7, 10^8 or 10^9SRBC per culture, then resuspended in fresh medium with freshly reclaimed, autologous PBL in 1:1 ratio and cultured as usual for six days with 10^8 SRBC/culture. All cultures containing pre-incubated cells gave a decreased response, those pre-cultured with 10^9 SRBC decreasing the fresh cell response the most. (Figure 13). Of particular interest was the suppressive capacity of those cells pre-incubated without antigen. It is not known whether the suppression observed with and without antigen can be due to the same mechanisms.

Similar results were obtained when the length of the pre-incubation was six days (Figure 14). In thse experiments those cells pre-incubated with 10^8 SRBC suppressed the fresh, autologous response the most. Again, a significant amount of suppression was observed after the addition of cells pre-incubated without antigen.

The next step in the investigation was to see which cell type was responsible for this suppression. All T and B cell separations for these experiments were performed on nylon wool columns.

When B cells were pre-incubated with 0, 10^8 or 10^9 SRBC for 24 hours there was less suppression of the autologous response than when unseparated PBL were pre-incubated. Although the statistical significance is greater for the B cell decrease than the PBL decrease (Figure 15), this may be due to the greater variation in responses from autologous lymphocytes when pre-incubated "B" cells are added to them. Variations in B cell purity are difficult to control and give less reproducible results. So when all the

data are used in a t-test, more significance may be observed by chance.

By adding pre-incubated T cells, the proportion of T and B cells in the responding culture is altered. Previous experiments (Table 8) show that the optimal response is obtained when 50% or 80% T cells are present. Figure 17 shows that when 10-20% of the responding culture consists of pre-incubated T cells, significant suppression is observed. Thus, it is not likely that the change in T to B cell ratio is the total cause of the observed suppression.

T cells pre-incubated alone, with or without antigen, completely inhibited the ability of freshly reclaimed, autologous PBL to form PFC (Figure 15). The same results were obtained when the length of pre-incubation was six days (Figure 16).

Adherent cells pre-incubated with or without antigen suppressed the autologous PBL response to approximately the same extent as B cells (Figure 18). No experiments to quantitate the suppressive capacity of B or adherent cells were performed so only the relative decreases in responses compared to controls can be compared (Figures 15 and 18).

Thus it appears that it is a T cell which can best be activated, by pre-incubation, into a suppressor cell in this system.

To quantitate the ability of the T cell population to suppress an autologous culture of PBL after pre-incubation, T cells which had been pre-incubated, or which were freshly reclaimed, were added to freshly reclaimed, autologous, PBL cultures (Figure 17).

The results show that pre-incubation of T cells with 10^9 SRBC induced a higher suppressive effect than pre-incubation in the absence of antigen. However, even in the absence of antigen pre-incubated T-cells expressed a significant amount of suppression. In both cases, the amount of suppression was dependent on the number of pre-incubated cells added, as seen in Figure 17 and Table 11. A suppressive index of 50% was reached

after adding 0.28 x 10^6 T cells, pre-incubated without antigen. In comparison, approximately 0.20 x 10^6 T cells pre-incubated with 10^9 SRBC were sufficient to cause the same suppressive effect. Since this study cannot address the possible mechanism of suppression, this difference may not be significant.

The observation that pre-incubation of lymphocytes in the absence of antigen can result in development of a suppressive effect is not unprecedented. Anaclerio et al (1979) reported that pre-culture of murine lymphocytes for 2-4 days induces aspecific suppressor cells to the uptake of tritiated thymidine by CON A stimulated lymphocytes in vitro. These suppressor cells were believed to be phagocytic cells, since treatment of the pre-cultured cells on plastic dishes abolished the effect. Burns et al (1975) reported that murine spleen cells cultured for four days non-specifically suppressed the humoral (PFC) responses of freshly prepared cells to a variety of antigens. These cells appear to be T lymphocytes and would be generated from either the short-lived, anti-thymocyte serum insensitive pool, or from the long lived, anti-thymocyte sensitive pool. Both these experiments used cells pre-cultured in FCS. However, the reasons for development of suppressor cells under these circumstances has never been sufficiently explained.

Pre-incubation may also induce suppressor cells to the cytotoxic response in man. Dubey et al (1979) found that these cells were contained in the monocyte/macrophage cell faction, they ingested latex, were esterase positive and did not rosette with SRBC. Suppression did not primarily take place via a cytotoxic effect against either the stimulator cells or the autologous responder cells.

In order to get some insight into factors possibly influencing the activation of suppressor cells or their precursors in the absence of anti-

genic stimulation, we conducted a series of experiments where some components of the culture medium were varied (Table 12).

As can be seen from this table, only serum-free media (RPMI-1640 or McCoy's) did not cause any substantial suppressive effect. RPMI-1640 with either pooled AB serum or autologous serum induced 100% suppression. These suppressor cells could not produce PFC when stimulated with 10^8 SRBC after the one day primary culture.

Since pre incubation in autologous serum also caused activation of suppressor T cells, one may postulate their activation by antibodies, or serum proteins non-immunoglobulin in nature, present in autologous serum. Antibodies formed to other antigens, the antigen itself, or antigen-antibody complexes, might activate these suppressor T cells.

To study one aspect of this question, sera from four systemic lupus erythematosus patients were studied, two who were positive for immune complexes by the Raji cell radioimmuno assay and two who were not (Theofilopoulos and Dixon, 1976). In this experiment T cells pre-incubated in any one of the sera were able to suppress the response of autologous PBL cultures (Table 12).

Suppressor T cells activated by pre-incubation with high doses of antigen have been studied in this system using SRBC (Heijnen et al, 1978; Shore et al, 1978) and using TNP-PAA as antigen (Galanaud, 1979).

Suppressor T cells can also be separated and studied in a test of cell mediated immunity (Lydyard and Hayward, 1980). In this system the T cells were pre-cultured with Con A. They may also be pre-cultured with purified protein derivative or streptokinase-streptodornase (Borkowksy and Valentine, 1979).

In future studies the technique described in this thesis might also be used to measure responses to synthetic polypeptides, proteins, and other antigens of defined amino acid or antigenic structure. As described in the introduction, genetic control of the immune responses to such antigens might be more readily dissected. Also, the responses, as measured by an indirect plaque assay, may be of interest, particularly in those individuals who do not make large numbers of Ig M plaques.

Suppressor cell activity has been reported in pathologic conditions although the mechanism of this action and their relationship to the disease state are unclear. Future studies using the system described here might show how suppressor cells function to regulate immune responses in vivo, both in the normal individual and in various altered immune states. The first step would be to compare quantitations of suppressor cells reported here to those obtainable from patients with autoimmune disease and immunodeficient states. Later experiments might study suppressor cell activity in all these groups in an assay measuring responses to Epstein-Barr virus or carcinoembryonic antigen. If technical problems such as antigen isolation and coupling of it to an erythrocyte could be overcome, the results might prove interesting.

Spontaneously arising suppressor cell activity in this system has been reported in patients with agammaglobulinemia. Lymphocytes from four of seven patients exhibited a dose dependent suppression of their PFC response to ovalbumin (Dosch and Gelfand, 1978). This activity was restricted to four patients who could generate PFC response in vitro. It is mediated by T lymphocytes which are Theophylline sensitive and bear receptors for Fc Ig G. These cells may play a role in preventing the

normal transition of pre-B cells to B cells in patients without B cells.

Froebel et all (1979) found reduced responses to CON A and other mitogens in patients with rheumatic disease. They postulated that this would be due to some ongoing immune responses being mounted against some as yet unknown disease associated antigen. These patients had normal numbers of T cells, and only slightly increased amounts of surface immunoglobulin. This suggests that some factor other than merely numbers of cells determines the decreased mitogen induced proliferation.

Mitogen induced PFC responses may also be reduced in multiple sclerosis. Paty et al (1978) reported that responses were most decreased in patients with the Dw 2 antigen.

Cells suppressing an MLC response have been reported in Hodgkin's disease (Engleman et al 1979) and in acute leukemia. (Bryan et al 1978). Engleman et al (1979) found that the suppressor cell could be a T cell or a non-T cell. It may be that radiation therapy induces or accentuates the detection of T suppressor cells.

Suppressor cells may be important in regulating responses to self antigens, so that self-tissue is not destroyed. Suppressor T cells may be altered or destroyed in autoimmune disease. (Dosch and Gelfand, 1976; Waldmann and Broder, 1977). Regulatory T cells may play a role in immune surveillance, controlling responses to self and non-self antigens. (Allison et al, 1971). Suppressor cell dysfunctions have also been postulated in the etiology of cancer (Pope et al, 1976).

Some suppressor T cells may have a beneficial effect of organ graft survival (Dossetor et al, 1979; Liburd et al, 1978).

Suppressor lymphocytes may also be important in human pregnancy (Kovithavongs and Dossetor, 1978).

The specificity of suppressor cells discussed in this thesis could be studied in two ways. (1) Allogeneic specificity: Do suppressor cells induced or measured in cultures of one individual also suppress the response to the same antigen by another individual? If so, is that suppression HLA-type related? (2) Antigenic specificity: Do suppressor cells induced in cultures containing SRBC also suppress the individual's response to horse, mouse, or ox erythrocytes? This might be slightly more difficult to show because of variation in antigenic dose response curves.

B. Conclusion

The following conclusions may be drawn from experiments presented in this thesis.

(1) The conditions for the optimal human PFC response *in vitro* to SRBC using this technique are: 1. six days of *in vitro* sensitization 2. 10^8 SRBC per culture, 3. 0.5×10^6 or 1.0×10^6 PBL per culture.

(2) Generation of an optimal PFC response *in vitro* to SRBC requires adherent cells, and an optimal ration of T:B lymphocytes.

(3) Freezing of PBL and reclaiming before culture may selectively inactivate a suppressor cell or suppressor cell precursor from fresh PBL populations.

(4) Pre-culturing of PBL, after being reclaimed from the frozen state, with or without antigen, may activate a suppressor cell which can act on autologous, reclaimed PBL cultured immediately after thawing.

(5) The most effective suppressor cell in this system is a T lymphocyte. It appears to be activated by some factor present in serum; exposure to SRBC may be less essential.

(6) The application of these *in vitro* studies to *in vivo* functions remains to be investigated.

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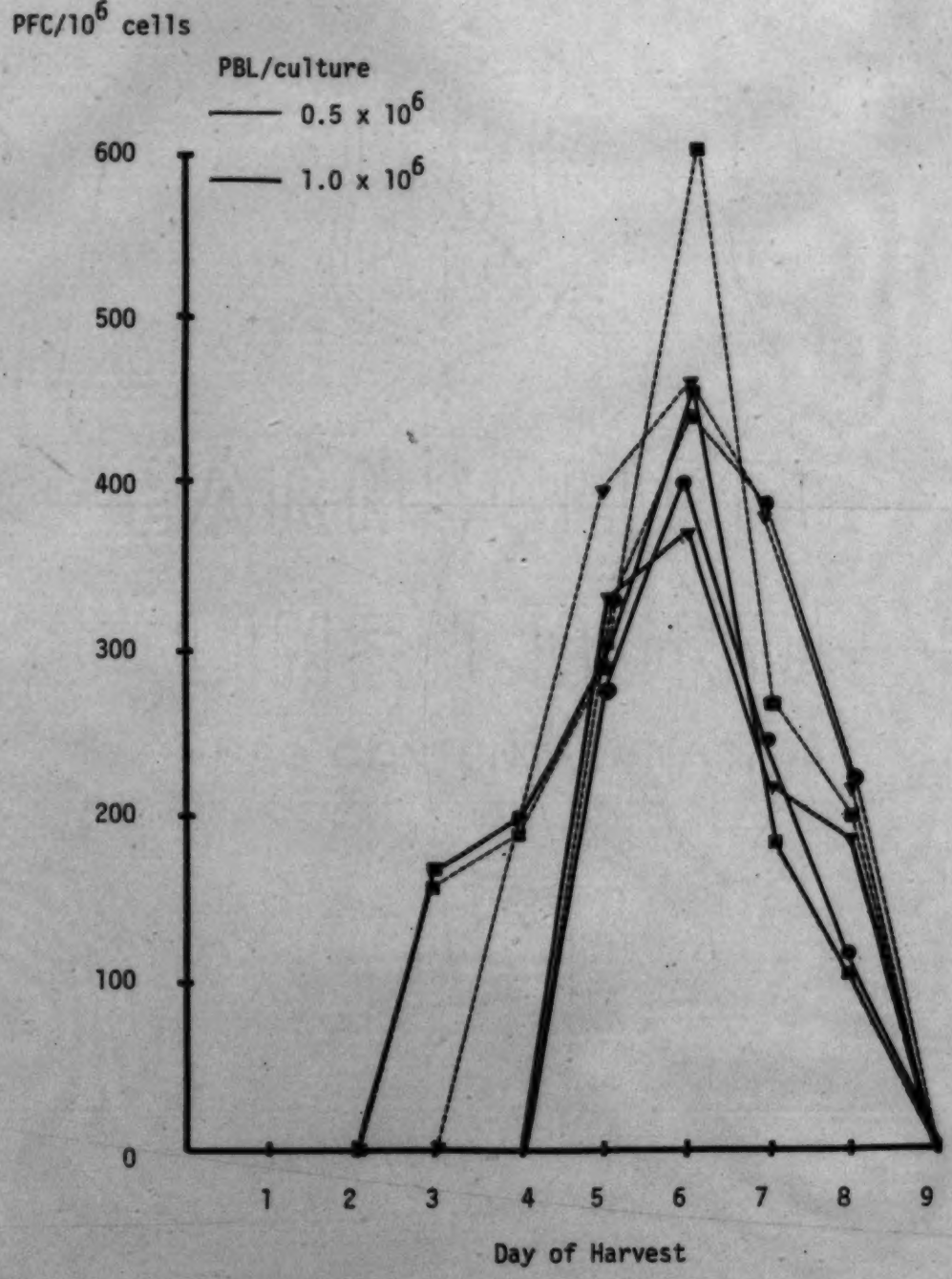
APPENDICES

Appendix 1. Kinetics of the PFC response. Replicate cultures from 3 individuals were harvested from day 1 to day 14. All responses were zero before day 3 and after day 8.

All cultures of 3.0×10^6 and of 7.0×10^6 cells from these individuals gave zero PFC. Circles, squares, and triangles each represent different individuals. All cultures were stimulated with 10^8 SRBC. Cultures stimulated with 10^7 or 10^9 SRBC gave zero PFC.

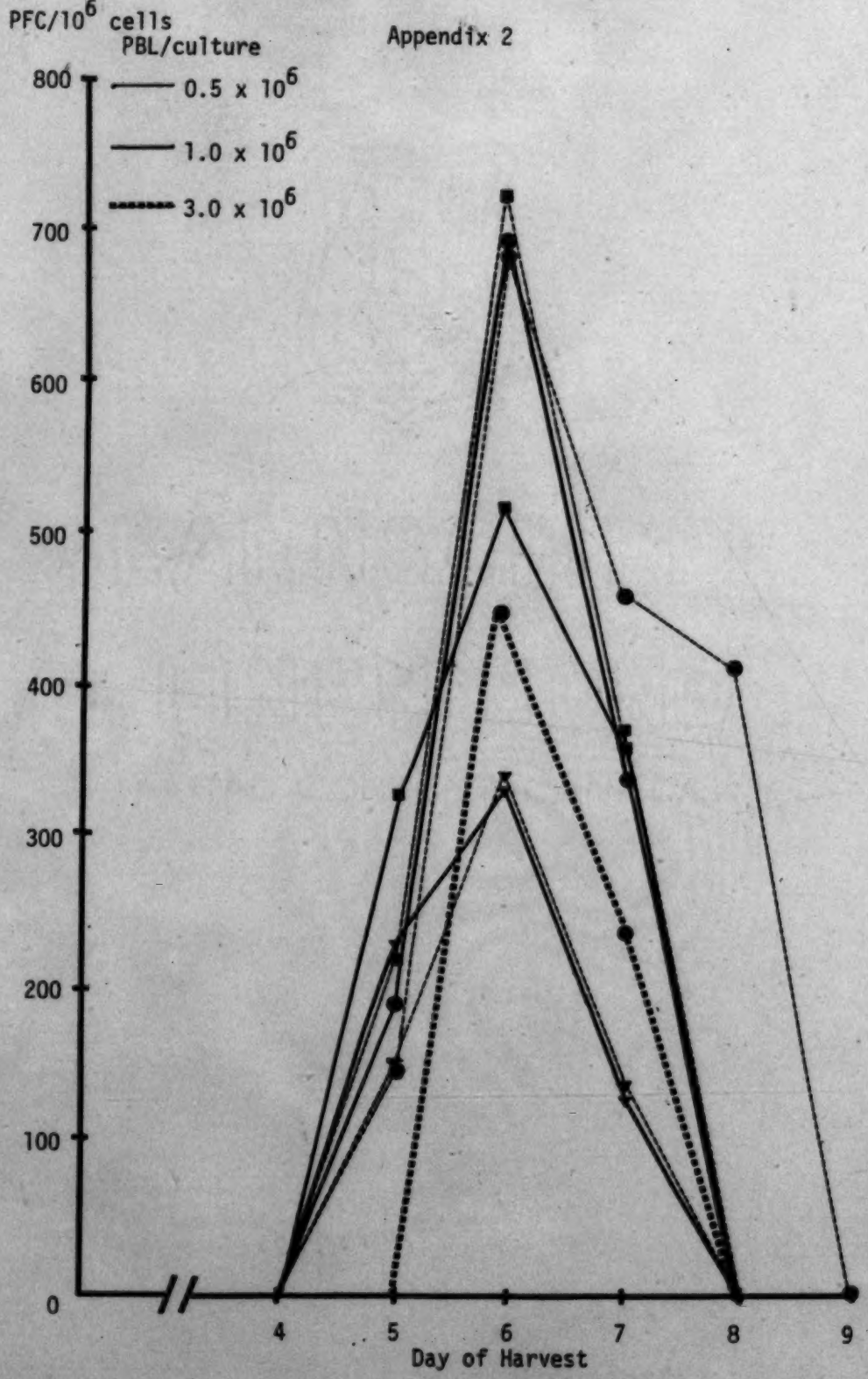
Appendix 1


PFC/10^6 cells
PBL/culture
0.5 x 10^6
1.0 x 10^6
600
500
400
300
200
100
0
1
2
3
4
5
6
7
8
9
Day of Harvest

Appendix 2. Kinetics of the PFC response. Replicate cultures from 3 individuals were harvested from day 1 to day 14. All responses were zero before day 5 and after day 8.

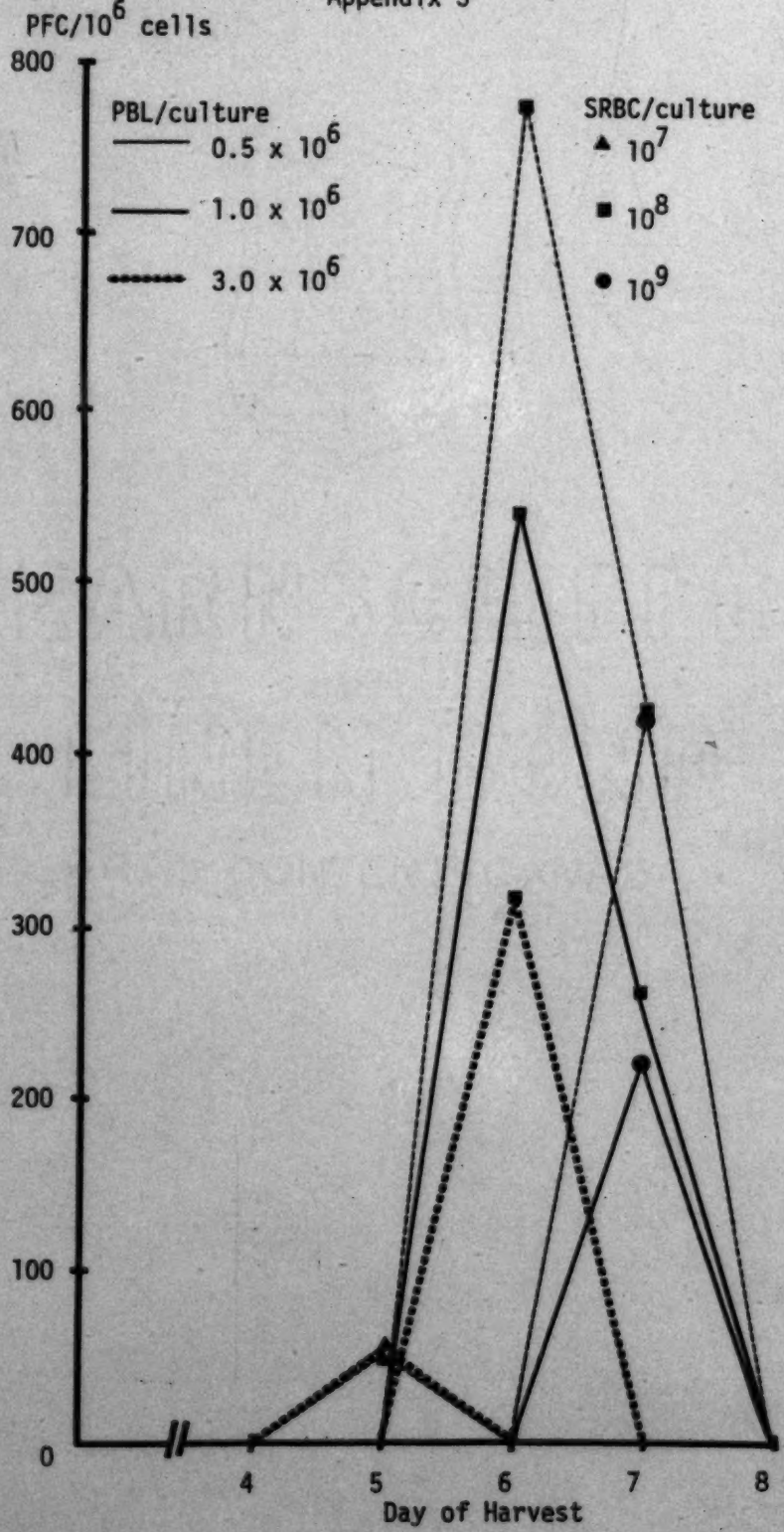
All cultures of 7.0×10^6 cells from these individuals gave zero PFC. Circles, squares, and triangles each represent different individuals. All cultures were stimulated with 10^8 SRBC. Cultures stimulated with 10^7 or 10^9 SRBC gave zero PFC.

Appendix 2


PFC/10^6 cells
PBL/culture
0.5 x 10^6
1.0 x 10^6
3.0 x 10^6
800
700
600
500
400
300
200
100
0
4
5
6
7
8
9
Day of Harvest

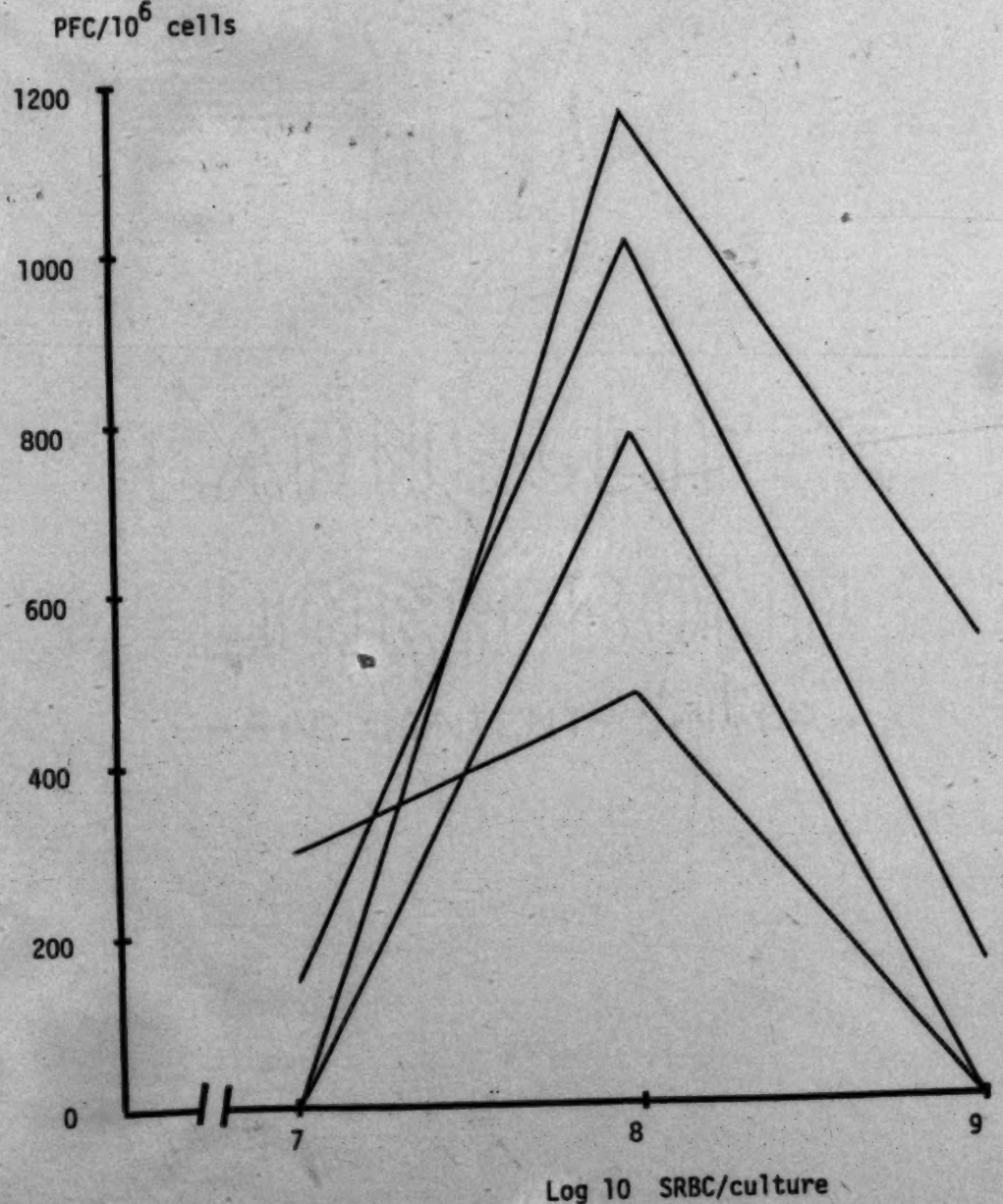
Appendix 3. Kinetics of the PFC response. Cultures from one individual were harvested from day 1 to day 14. All responses were zero before day 5 and after day 8.

Appendix 3


PFC/10^6 cells
800
700
600
500
400
300
200
100
0
PBL/culture
0.5 x 10^6
1.0 x 10^6
3.0 x 10^6
SRBC/culture
10^7
10^8
10^9
4
5
6
7
8
Day of Harvest

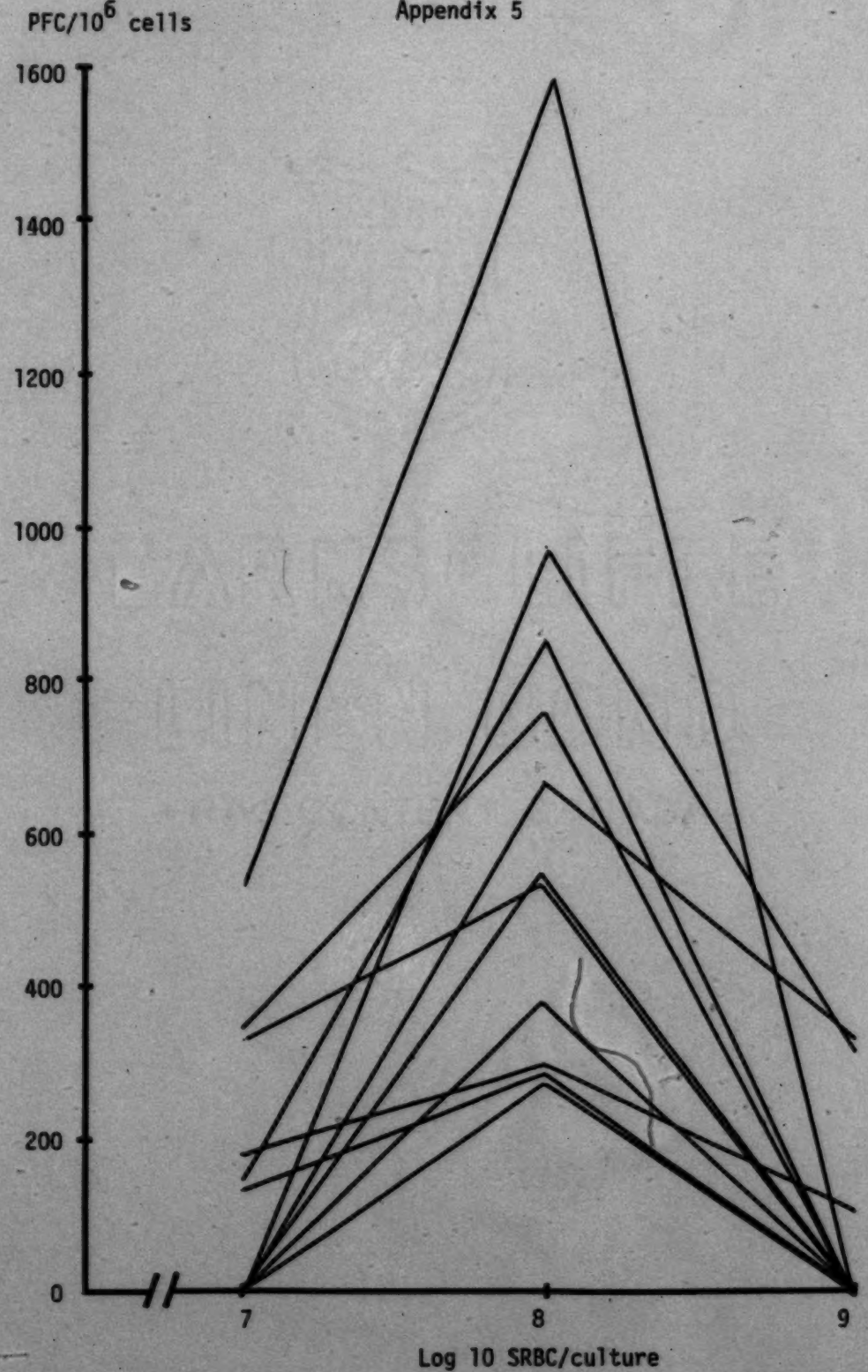
Appendix 4. Effect of varying antigen dose on the PFC response. All cultures were harvested on day 6 and contained 0.25×10^6 cells. Eight individuals were tested and four gave zero PFC at all of the antigen doses.

Appendix 4


PFC/10^6 cells
1200
1000
800
600
400
200
0
7
8
9
Log 10 SRBC/culture

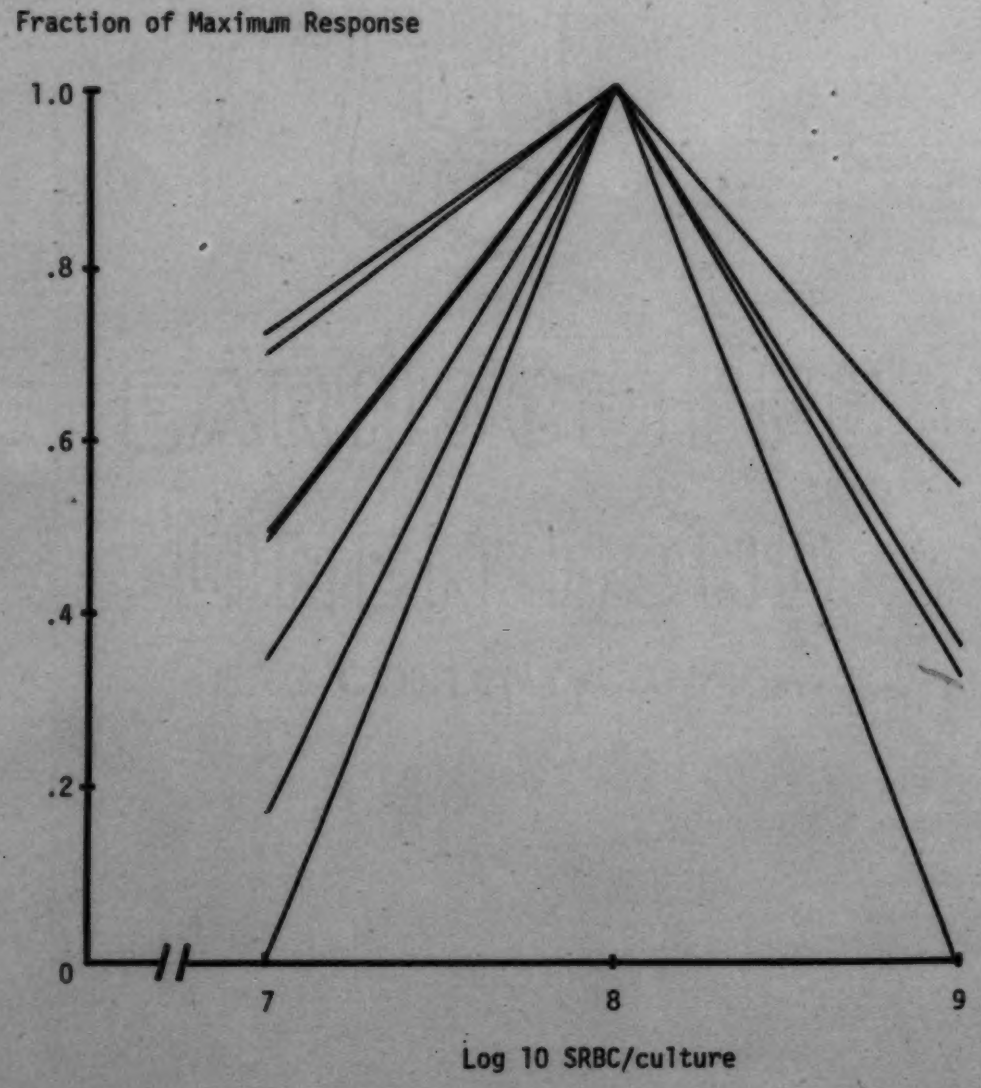
Appendix 5. Effect of varying antigen dose on the PFC response. All cultures were harvested on day 6 and contained 0.5 x 10^6 cells. Eleven individuals were tested at each of the three antigen doses.

Appendix 5


PFC/10^6 cells
1600
1400
1200
1000
800
600
400
200
0
7
8
9
Log 10 SRBC/culture

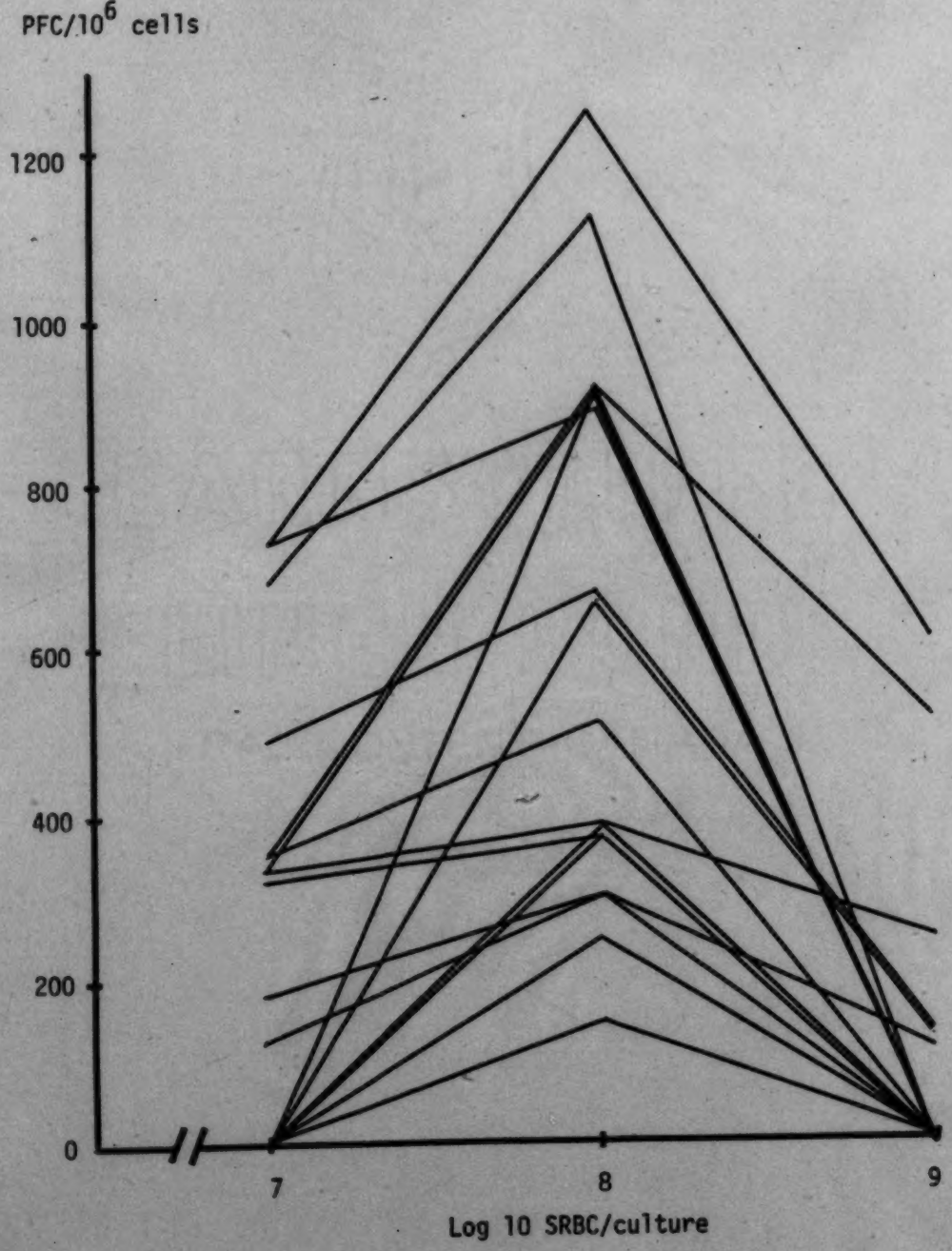
Appendix 6. Effect of varying antigen dose on the PFC response. The fraction of the maximum response is plotted for each of the 11 individuals in appendix 5. 5 individuals gave zero PFC with 10^7 SRBC, and 8 individuals gave zero PFC with 10^9 SRBC.

Appendix 6


Fraction of Maximum Response
1.0
.8
.6
.4
.2
0
7
8
9
Log 10 SRBC/culture

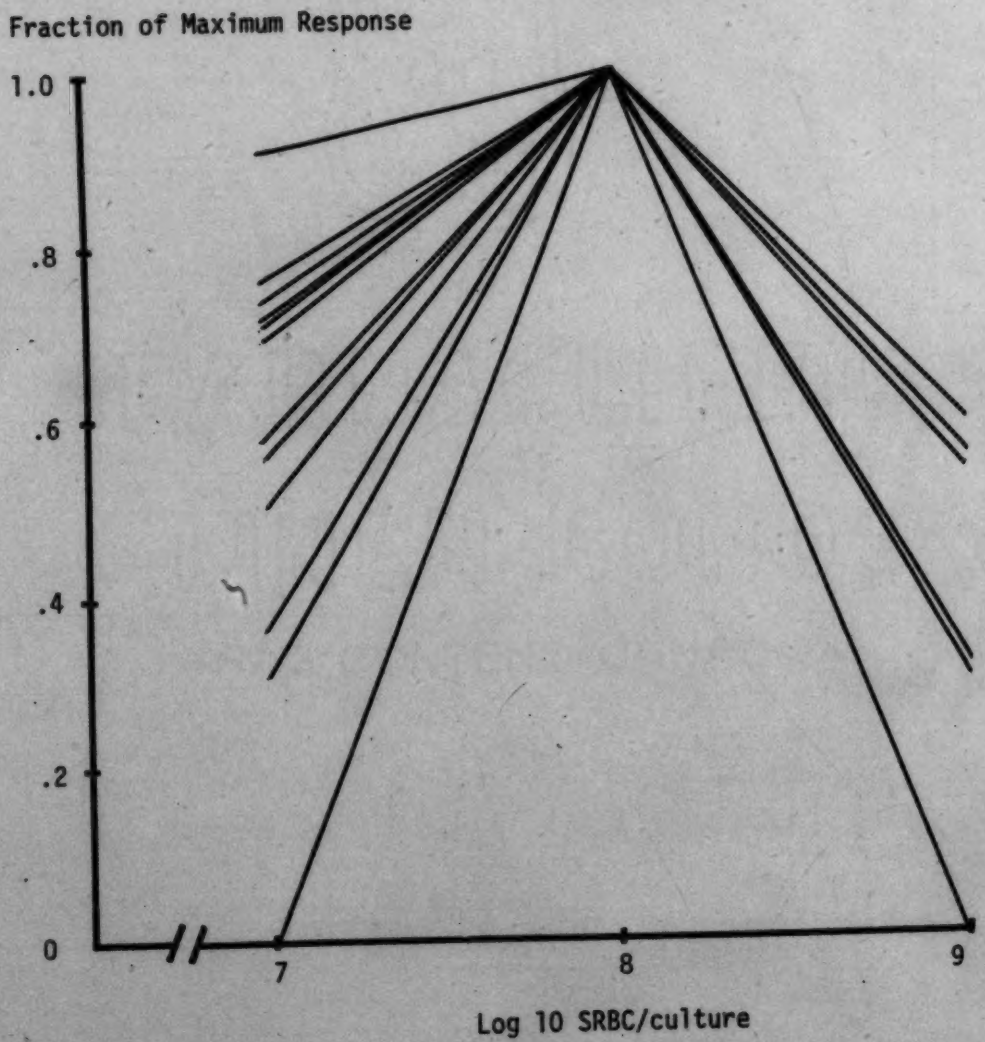
Appendix 7. Effect of varying antigen dose on the PFC response. All cultures were harvested on day 6 and contained 1.0 x 10^6 cells. Seventeen individuals were tested at each of the three antigen doses.

Appendix 7


PFC/10^6 cells
1200
1000
800
600
400
200
0
7
8
9
Log 10 SRBC/culture

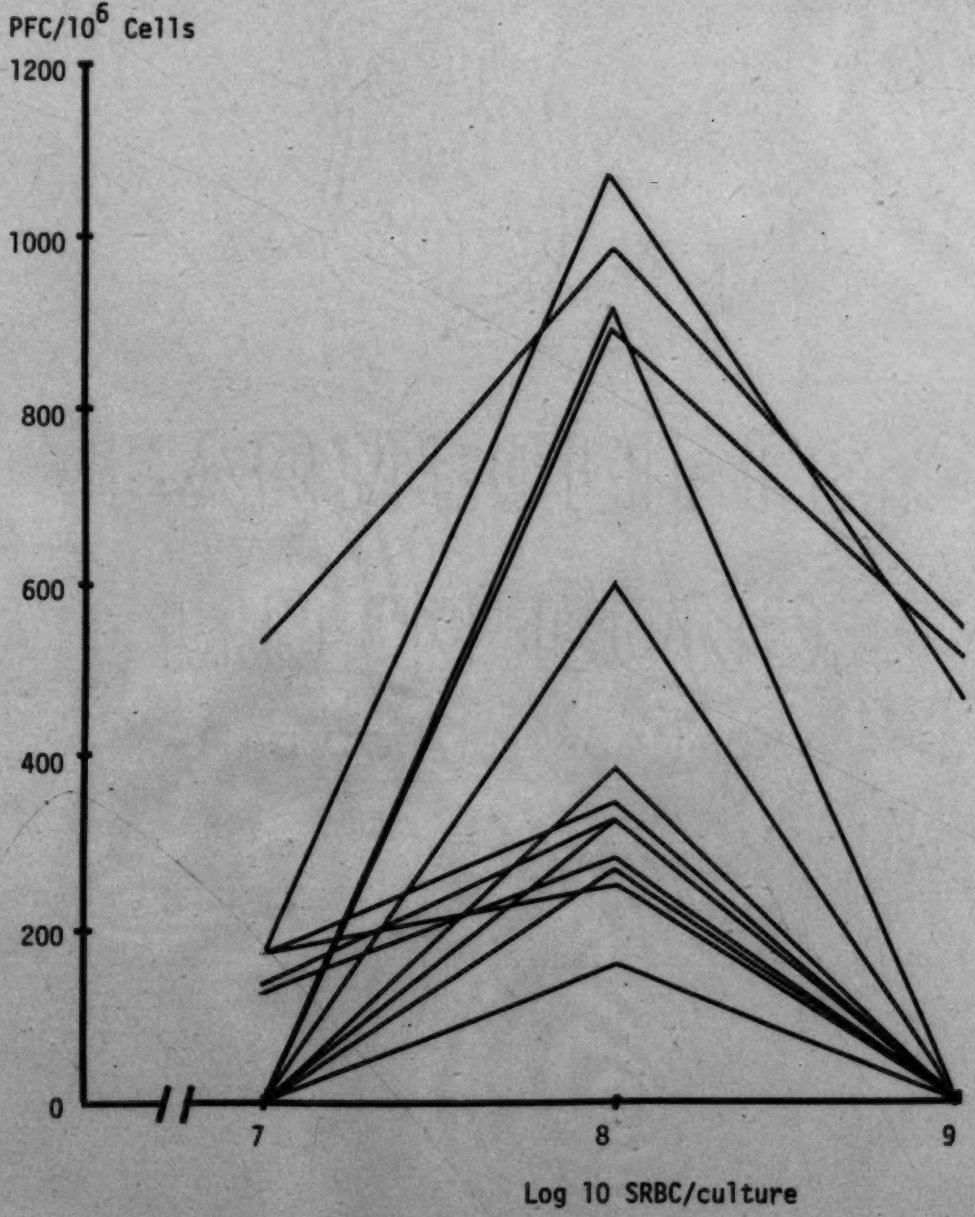
Appendix 8. Effect of varying antigen dose on the PFC response. The fraction of the maximum response is plotted for each of the 17 individuals in appendix 7. 6 individuals gave zero PFC with 10^7 SRBC, and 10 individuals gave zero PFC with 10^9 SRBC.

Appendix 8.


Fraction of Maximum Response
1.0
.8
.6
.4
.2
0
7
8
9
Log 10 SRBC/culture

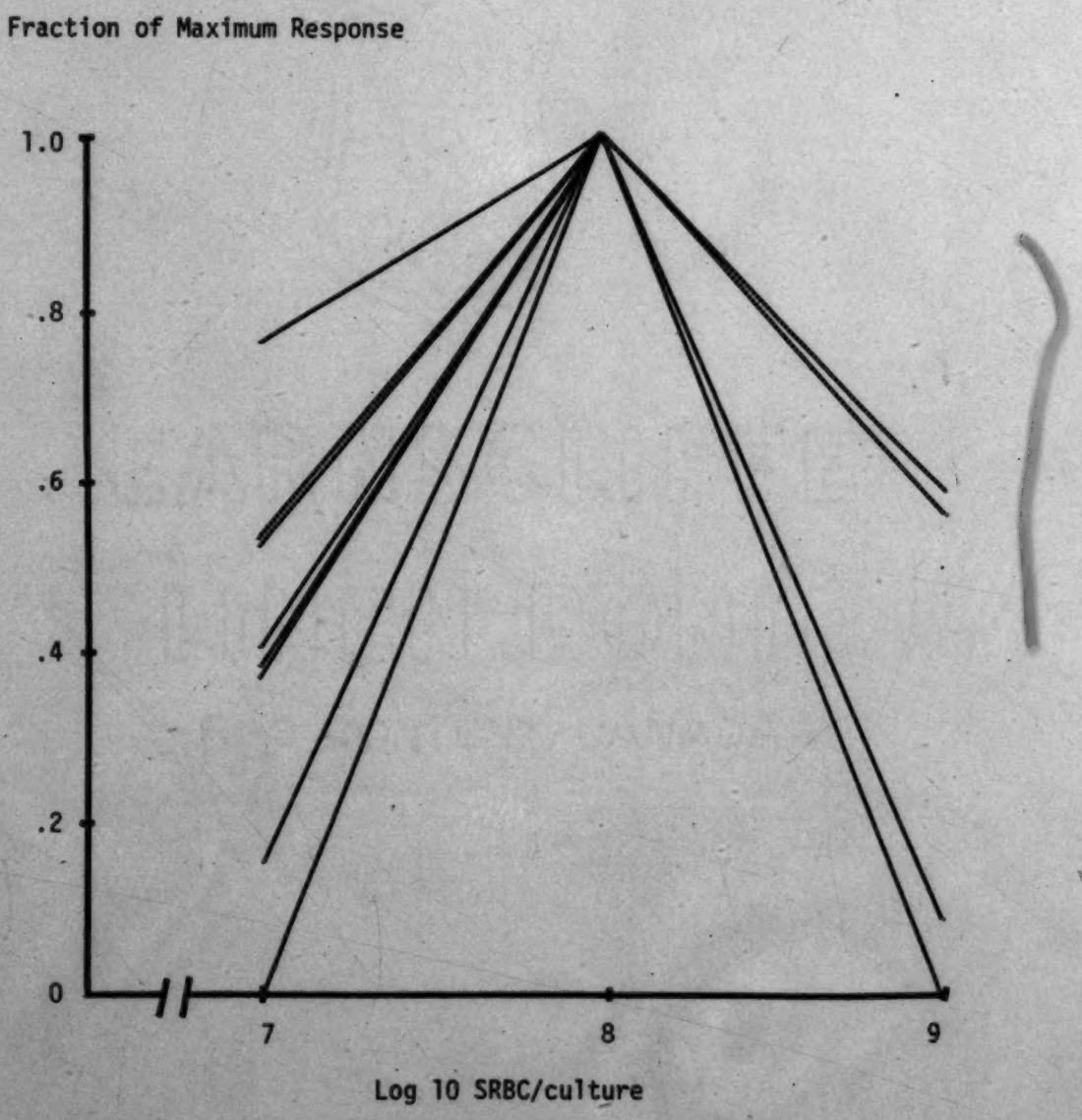
Appendix 9. Effect of varying antigen dose on the PFC response. All cultures were harvested on day 6 and contained 3.0×10^6 cells. Fourteen individuals were tested at each of the three antigen doses.

Appendix 9


PFC/10^6 Cells
1200
1000
800
600
400
200
0
7
8
9
Log 10 SRBC/culture

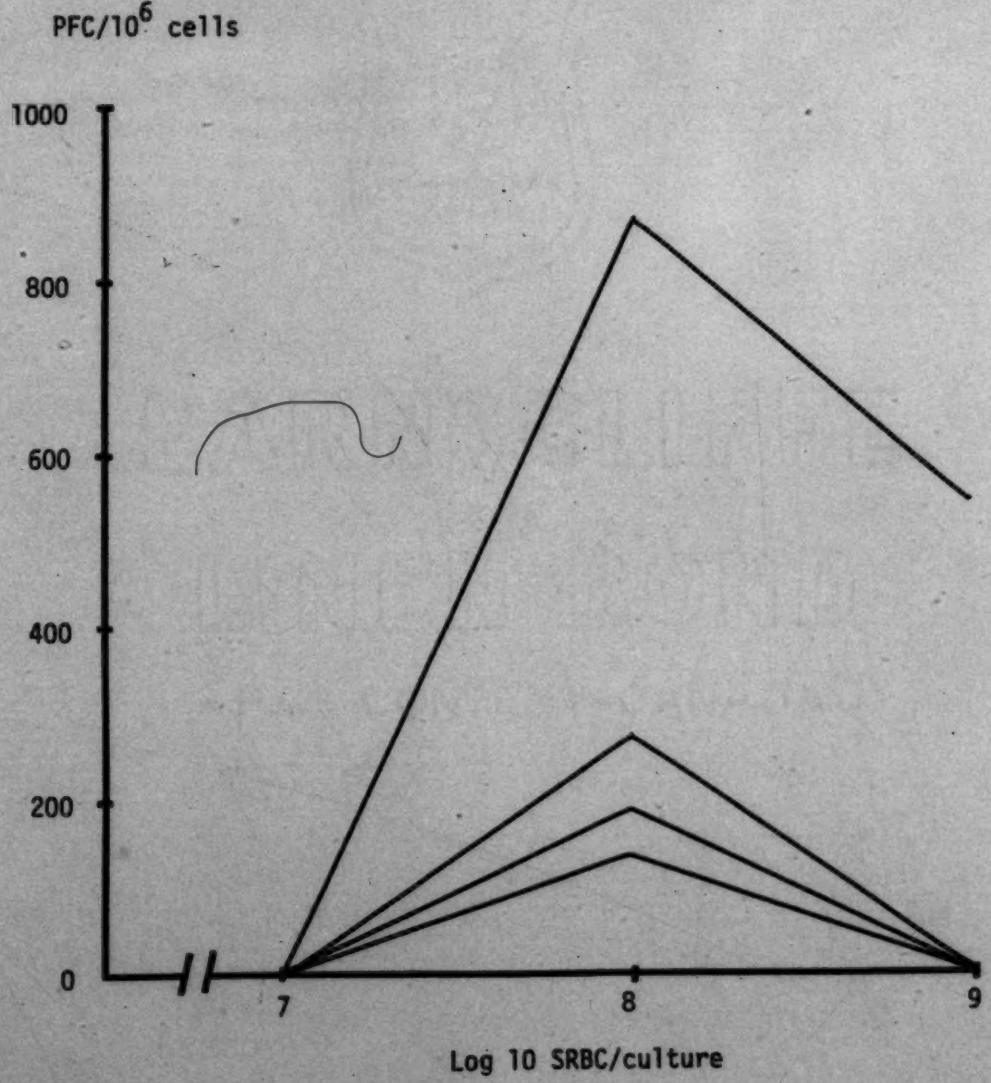
Appendix 10. Effect of varying antigen dose on the PFC response. The fraction of the maximum response is plotted for each experiment in appendix 9. 7 individuals gave zero PFC with 10^7 SRBC, and 9 individuals gave zero PFC with 10^9 SRBC.

Appendix 10


Fraction of Maximum Response
1.0
.8
.6
.4
.2
0
7
8
9
Log 10 SRBC/culture

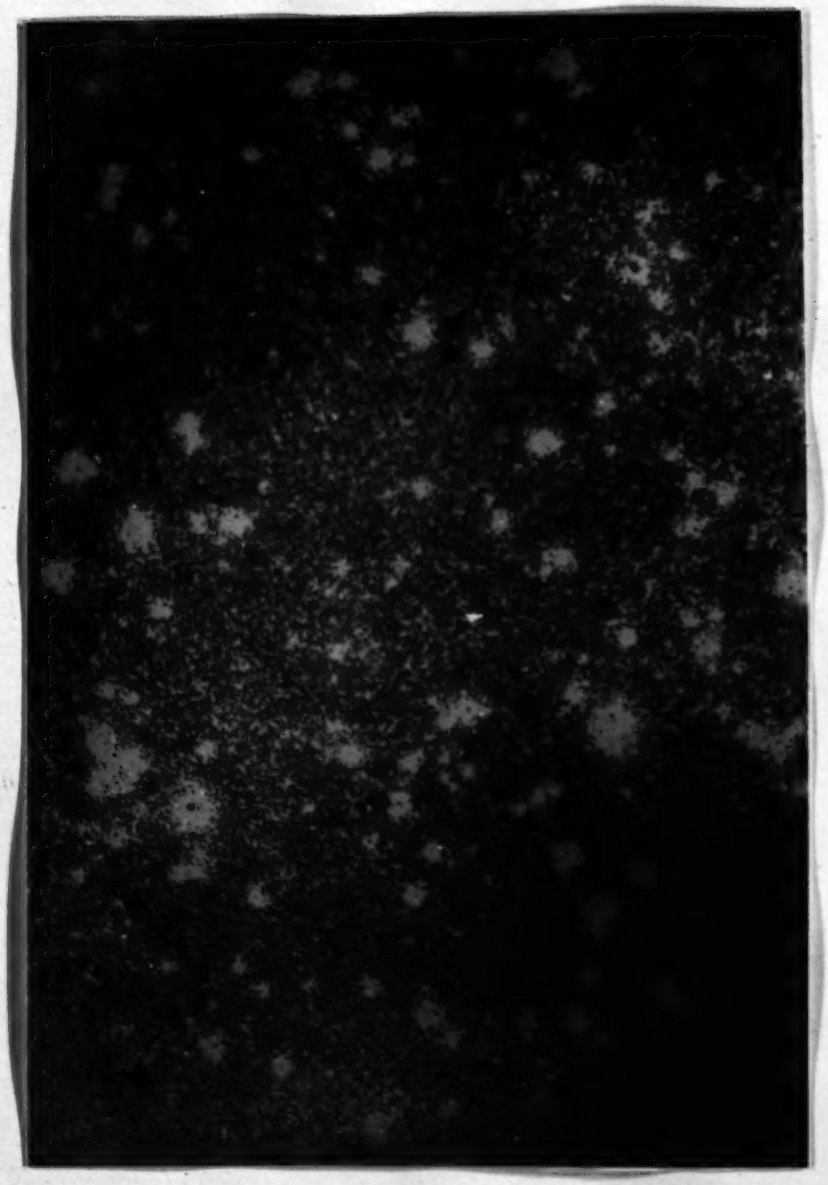
Appendix 11. Effect of varying antigen dose on the PFC response. All cultures were harvested on day 6 and contained 7.0 x 10^6 cells. Seventeen individuals were tested at each of the three antigen doses. Thirteen individuals gave zero PFC at all of the antigen doses.

Appendix 11



Appendix 12. Plaques seen in one well under 25 x magnification. Half a million lymphocytes were stimulated with 10^8 SRBC.

Appendix 12



Appendix 13. Plaques seen in one well under 100 x magnification. Half a million lymphocytes were stimulated with 10^8 SRBC.

Appendix 13